Changing the balance in apoptosis

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Changing the balance in apoptosis: key to enhance cisplatin sensitivity of testicular cancer

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Content

Chapter 1

General introduction & Scope of this thesis

General introduction

Testicular cancers represent the most frequent solid malignant tumor in men 20-40 years of age and the incidence of testicular cancer has been arising world-wide (1). Based on histology these tumors can be divided in seminomatous and non-seminomatous testicular cancer. Non-seminomas tend to metastasize more widely and are less sensitive to radiotherapy than seminomatous testicular cancer. This has consequences for prognosis and treatment strategies. Non-seminomas display various stages of differentiation, ranging from the undifferentiated embryonal carcinoma (EC) to more differentiated extra-embryonic components like yolk sac carcinomas (YS) and choriocarcinomas (Chc) to the highly differentiated teratoma (T) component (2).

Platinum-based chemotherapy is the cornerstone of treatment of many cancers types and used as first-line therapy for testicular, bladder, lung, ovarian cancers. In case of bladder, lung and ovarian cancer the initial response to platinum-based chemotherapy is high, however is in the majority of these patients of limited duration. Most patients eventually will relapse with platinum-resistant disease. In contrast, testicular cancer is considered to be a paradigm of a platinum-sensitive solid tumor. Since the introduction of cisplatin in the mid 1970s there is an enormous improvement in outcome and survival of testicular cancer patients even in case of extensive metastatic disease (3). Further improvement of the cisplatin-based regimens has been executed in the years following the initial success (4,5). Bleomycin has proven to be an essential component of the cisplatin-based chemotherapy regimens (6), leading to the current standard systemic treatment for testicular cancer BEP combination chemotherapy (bleomycin, etoposide and cisplatin).

Patients with disseminated testicular cancer are classified according to the International Germ Cell Consensus Classification into three prognosis groups with good, intermediate and poor prognosis, with a 5-year survival rate after treatment with cisplatin combination chemotherapy of 91%, 79% and 48%, respectively (7). Despite the overall treatment success, about 20~50% of the testicular cancer patients with extensive metastatic spread belonging to the intermediate or poor risk group will not achieve a durable remission after initial treatment and will eventually die from their disease (5).

Cisplatin becomes activated intracellularly by the aquation of one or both of the two chloride ligands before it covalently binds to DNA (8), while bleomycin has DNA-cleaving activity when it is in complex with an oxygenated iron (9). Upon treatment with either cisplatin or bleomycin, lethal DNA adducts are formed (8,9), which cause arrest of the DNA replication fork, and distortions in DNA, including unwinding and bending, and which are recognized by several cellular proteins (8,10). This process activates various signal-transduction pathways involved in DNA-damage recognition followed by cell-cycle arrest and repair or apoptosis (8,10,11). Resistance to cisplatin or bleomycin can be the result of reduced drug uptake, increased drug export, intracellular detoxification, enhanced efficiency of DNA repair systems (8,10-13) and defects in the apoptotic pathway (14).

Apoptosis, also known as programmed cell death, is an active cellular process, characterized by biochemical and morphological changes (Figure 1). Apoptosis is involved in development and homeostasis of normal tissues (15), among which normal testis. During spermatogenesis, apoptosis occurs in the testis as an important physiological mechanism to adjust germ cell numbers to that of the supporting Sertoli cells and to ensure quality control (16,17). A central component of the apoptotic machinery is a proteolytic system that involves caspases, a family of proteases which activity eventually leads to the cleavage of a set of

downstream proteins, resulting in the overall disassembly of the cell (Figure 1). Apoptosis can be induced by two major apoptosis signaling pathways namely the "extrinsic" death receptor pathway and the "intrinsic" mitochondrial pathway (15) (Figure 1). The tumor suppressor protein p53 plays a central role in the response to DNA-damage, induced by chemotherapeutic drugs, and can activate both the extrinsic and intrinsic death signaling pathways (14) (Figure 1).

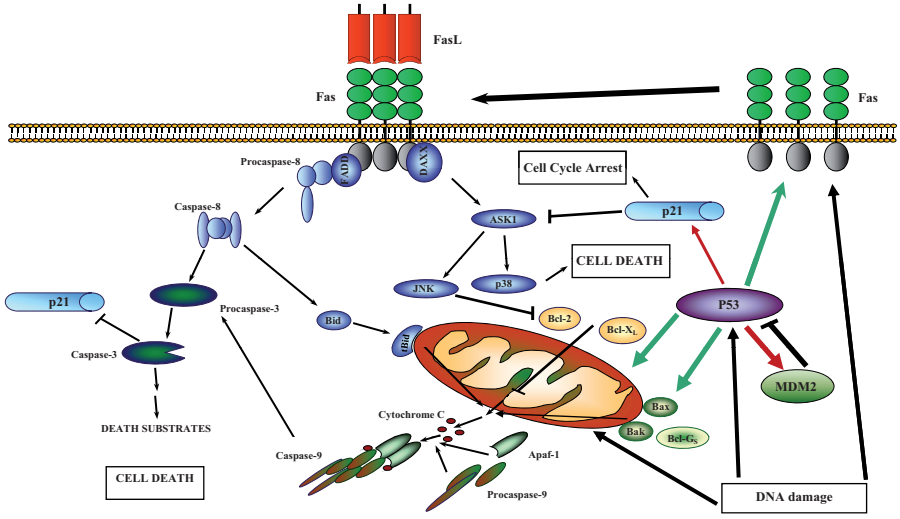

Figure 1. Apoptosis or programmed cell death is an active cellular process, characterized by biochemical and morphological changes. The tumor suppressor protein p53 plays a central role in the response to DNA-damage, induced by chemotherapeutic drugs such as cisplatin, and can activate both the extrinsic apoptosis pathway (including Fas/FasL) and intrinsic (mitochondrial) apoptosis pathway; e.g. Bax and Bak. Activation of p53 leads to upregulation of pro-apototic genes (green lines) and anti-apoptotic genes (red lines). These activate various signal-transduction pathways involved in DNA-damage recognition following cell-cycle arrest and repair (via p21) or apoptosis. Upon severe DNA-damage pro-apoptotic proteins will activate a proteolytic system that involves caspases, a family of proteases which eventually leads to the cleavage of a downstream set of proteins, resulting in the overall disassembly of the cell. Further explanation is provided in the text. Figure modified from Di Pietro et al., 2005 and Spierings et al., 2003; Refs (22,23).

P53 is a tumor suppressor protein with a dual role in stress response by transactivating genes that induce apoptosis, such as Fas (death receptor pathway), as well as genes that induce cell-cycle arrest, such as *CDKN1A* (encoding p21$^{cip1/waf1}$). In addition, p53 regulates its negative feed-back regulator, MDM2, which interferes with p53 transactivation activity and targets it for degradation (18) (Figure 1). *P53* is the most frequently mutated gene in human cancers (19,20), but surprisingly not in testicular cancer. Only in a small proportion of testicular cancer patients, mutations in *TP53* were found (21). In these patients, mutant p53 led to cisplatin resistance of the cancer by affecting the downstream apoptotic pathway (21). Therefore, the presence of wild-type p53 might explain the sensitivity to cisplatin (22,23), especially since it is expressed at high levels in the majority of TCs (24-27). Though, others have studied chemo-sensitivity of testicular cancer cell lines and failed to support a role for wild-type p53 (28-30). Recently, it has been reported that cisplatin treatment of TC cells

resulted in enhanced levels of p53 and MDM2, activation of the Fas apoptotic pathway, and induction of apoptosis, while the expression levels of p21 were hardly affected (25,31). These results demonstrated the involvement of the Fas apoptotic pathway in cisplatin-induced apoptosis in testicular cancer cells (25,31). However, it is not known why cisplatin-induced p53 activation did not result in activation of p21. This suggests that changes in the balance of key-players in the cisplatin-induced p53-mediated apoptotic pathway might determine fate of testicular cancer cells treated with cisplatin. Deciphering the complex regulation of these molecular determinants of cisplatin-sensitivity and -resistance in testicular cancer treatment, may provide a way to improve chemotherapy sensitivity in resistant TCs and other solid tumors.

Scope of the thesis

Though p53 is one of the most studied proteins, the precise effect of wild-type p53, and downstream targets, on the response to DNA-damage in testicular cancer is still not clear. Therefore, this thesis mainly focuses on the functionality of wild-type p53 and downstream targets Fas, MDM2 and p21 and their role in determining the response to cisplatin-induced apoptosis in testicular cancer.

Recent studies have demonstrated that enhanced p21 expression protects TC/EC cells against Fas-induced apoptosis (25), though the role of p21 in protecting against cisplatin-induced apoptosis in not clear. In **Chapter 2** we, therefore, determined the role of p21 in cisplatin-induced apoptosis, using cisplatin-sensitive and -resistant TC cell lines. Expression levels of p21 and *CDKN1A* and the sub-cellular localization of p21 at baseline and after treatment with cisplatin were investigated. To demonstrate the importance of cytoplasmic p21 in the protection against apoptosis-induction, p21 was suppressed in the TC cells. Moreover, cytoplasmic p21 was ectopically over-expressed in the cisplatin-sensitive Tera cells. Next, targets of cytoplasmic p21 were identified by co-immunoprecipitation and their involvement in cisplatin sensitivity was investigated. Mechanism involved in p21 cytoplasmic localization and strategies to relocate cytoplasmic p21 to the nucleus were explored. Furthermore, clinical relevance of cytoplasmic p21 expression in EC components from chemo-sensitive and -refractory TC patients was studied in detail. Finally, we dissected the pathway involved in the regulation of p21 expression levels, i.e. the role of Oct4 in regulating p21 expression via the miR-106b seed family in EC.

Several reports have been studying chemo-sensitivity of human TC cell lines in relation to wild-type p53 expression, however with contradicting results. In **Chapter 3** we determined in detail the role of the p53-dependent and p53-independent apoptotic pathway in cisplatin sensitivity and resistance in a model resembling the clinically observed variation in chemo-sensitivity between testicular cancers. We compared p53-dependent cellular and molecular changes with respect to p21, MDM2 and Fas at baseline and after cisplatin treatment in two cisplatin-sensitive cell lines (833KE and Tera), a subline of Tera with acquired resistance to cisplatin (Tera-CP) and two intrinsic cisplatin-resistant cell lines (Scha and 2102EP). Furthermore, the effect on cisplatin-induced apoptosis was studied after p53 suppression.

P53 interacting proteins, such as MDM2, are important regulators of wild-type p53 functional activity (32,33). MDM2 interferes with p53 transactivation and targets p53 for degradation (18). High levels of wild-type p53 are frequently observed in TC and correlate with high MDM2 expression levels (29,34,35). A tight regulation of p53 by MDM2 may explain the lack of *TP53* mutations in TC. Various reports have focused on the p53 pathway

to explain the chemo-sensitivity of TCs. A recent study demonstrated that expression of p53 and MDM2 did not predict for cisplatin sensitivity, though the presence and functionality of the p53-MDM2 complex following cisplatin treatment was not assessed (29). In **Chapter 4**, we therefore extensively explored the role of p53 and MDM2 in cisplatin-induced apoptosis using cisplatin-sensitive and -resistant TC cell lines. Next to expression levels, the sub-cellular localization of p53 and MDM2 and p53-MDM2 complex formation after treatment with cisplatin was determined. The biological importance of MDM2-p53 complexes in relation to hyper-activation of the p53 pathway and apoptosis-induction was further investigated using the MDM2 small molecule antagonist Nutlin-3 as single agent and in combination with cisplatin. Finally, we determined if the Fas death receptor pathway is a key factor in Nutlin-3 induced apoptosis.

In **Chapter 5**, an overview of the literature and new insights obtained in this thesis on the potential pathways responsible for the extreme sensitivity of testicular cancers to cisplatin-based chemotherapy are presented. This review takes into account p53-activated genes, such as *CDKN1A* (p21), *FAS* and *MDM2* (the negative feed-back regulator of p53) and the role of these genes in sensitivity to cisplatin-induced apoptosis. Therapeutic options to target these key-players are discussed in more detail as well.

Recently, we showed that the somatic homozygous variant G/G for the single nucleotide polymorphism (SNP) A1450G in bleomycin hydrolase (BLMH) was associated with reduced survival and a higher prevalence of early relapses in TC patients treated with bleomycin, etoposide and cisplatin chemotherapy (36). BLMH metabolically inactivates bleomycin (37) and this SNP in BLMH is thought to be involved in controlling the enzymatic activity (38,39). Of interest, elevated expression of BLMH was observed in bleomycin-resistant human tumor cell lines (12,13), suggesting that activity and/or levels of BLMH influence toxicity and antitumor efficacy of bleomycin. Therefore we investigated in **Chapter 6** the sensitivity of TC cell lines to bleomycin and cisplatin, and related the sensitivity to the BLMH genotype and expression levels of BLMH. Moreover, we suppressed BLMH in these cells with siRNA, to investigate the relation between BMLH level and the cytotoxic response to bleomycin or cisplatin.

In **Chapter 7**, results and findings from this thesis are summarized. Potential future perspectives and therapeutic approaches are discussed.

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Chapter 2

Cytoplasmic p21 expression levels determine cisplatin-resistance in human testicular cancer

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Abstract

Platinum-based chemotherapies such as cisplatin are used as first-line treatment for many cancers. Although there is often a high initial responsiveness, the majority of patients eventually relapse with platinum-resistant disease. For example, a subset of testicular cancer patients still die even though testicular cancer is considered a paradigm of cisplatin-sensitive solid tumors, but the mechanisms of chemo-resistance remain elusive. Here, we have shown that one key determinant of cisplatin-resistance in testicular embryonal carcinoma (EC) is high cytoplasmic expression of the cyclin-dependent kinase (CDK) inhibitor p21. The EC component of the majority of refractory testicular cancer patients exhibited high cytoplasmic p21 expression, which protected EC cell lines against cisplatin-induced apoptosis via CDK2 inhibition. Localization of p21 in the cytoplasm was critical for cisplatin-resistance since relocalization of p21 to the nucleus by Akt inhibition sensitized EC cell lines to cisplatin. We also demonstrated in EC cell lines and human tumor tissue that cytoplasmic high p21 expression and cisplatin-resistance of EC were inversely associated with the expression of Oct4 and miR-106b seed family members. Thus, targeting cytoplasmic p21, including by modulation of the Oct4/miR-106b/p21 pathway, may offer new strategies for the treatment of chemo-resistant testicular and other types of cancer.

Introduction

In testicular cancer, even in case of extensive metastatic disease an enormous decrease in mortality has been observed with the introduction and use of highly effective cisplatin-containing chemotherapy schemes (1-2). Therefore, testicular cancer (TC) is considered the paradigm for curative disease. Despite the overall treatment success, about 20-50% of the TC patients with extensive metastatic spread belonging to the intermediate or poor risk group will not achieve a durable complete remission after initial treatment and will eventually die from this disease (3). The molecular basis for resistance, however, remains obscure.

A major role in the response to chemotherapeutic drugs and the execution of apoptosis has been ascribed to wild type p53 (4). In only a small proportion of TCs, mutations in p53 effect the downstream apoptotic pathway and lead to resistance (5), in particular as wild type p53 is expressed at high levels in the majority of TCs (6-9). p53 is a tumor suppressor protein with a dual role in stress response by transactivation of genes that induce apoptosis, such as *FAS*, as well as genes that induce cell-cycle arrest, such as *CDKN1A* (encoding $p21^{cip1/waf1}$). Interestingly, the cyclin dependent kinase inhibitor $p21^{cip1/waf1}$ (p21) has been shown to inhibit apoptosis (10-13). Remarkably, several studies have demonstrated that p21 protein and *CDKN1A* mRNA expression are abundantly expressed in more differentiated TCs, like mature teratoma (14-15). Worthy of note, these teratomas are resistant to cisplatin-based chemotherapy (8,15-17). In contrast, p21 is almost not detectable in seminomas and embryonal carcinomas (EC) that are predominantly sensitive to cisplatin (9,14,18).

In human TC/EC cell lines, similar to patients, cisplatin proved to be an extremely cytotoxic drug, inducing massive apoptosis (19-22). Cisplatin treatment of EC cells resulted in enhanced levels of p53 and MDM2, activation of the Fas apoptotic pathway, and induction of apoptosis, while the expression levels of p21 were almost not affected (7,20). In contrast, gamma-irradiation induced p53 and MDM2 levels and a massive induction of cytoplasmic p21 without inducing apoptosis or cell cycle arrest in EC cells (7). These results suggested an important role for cytoplasmic p21 in preventing DNA damage-induced apoptosis in EC cells.

Materials and Methods

Cell Lines & Reagents. A well defined panel of cisplatin-sensitive and cisplatin-resistant human EC cell lines, all expressing wild type p53 were used (7,20,22-24). The 2102EP cell line was obtained from Dr. L. Looijenga (Department of Pathology, Erasmus Medical Center Rotterdam, the Netherlands). Tera, Tera-CP, 2102EP, Scha and 833KE and the human breast carcinoma cell line MCF-7 (used as a control) were cultured in RPMI 1640 medium (Gibco, Invitrogen) supplemented with 10% FCS (Sanbio) at 37°C in a humidified atmosphere with 5% CO2. Cisplatin was purchased from Bristol-Myers Co., LY294002 from Cell Signalling and Triciribine from Biomol.

Drug Sensitivity Assay & Apoptosis. Drug sensitivity testing was performed as described previously (20,24). Acridine orange fluorescent staining of nuclei was used to distinguish apoptotic from vital cells as described previously (7,20,25).

Western Blotting & Preparation of fractionated proteins. Lysates were examined by WB as described previously (7,20). Nuclear and cytosolic protein fractions were prepared as described previously (7). The following antibodies were used: mouse anti p21 (F5, Santa Cruz), mouse anti p53 (DO-1, Santa Cruz), mouse anti b-Actin (MP Biomedicals), mouse anti pRB (IF8, Santa Cruz), rabbit anti-Parp (Roche Diagnostics), mouse anti ASK1 (F9, Santa Cruz), goat anti CDK2 (M2, Santa Cruz), rabbit anti p-p21 (Thr145, Santa Cruz), rabbit anti p-Akt/Akt (Cell Signalling), goat anti Oct4 (C20, Santa Cruz) and rabbit anti caspase 3 (cell Signalling). The antibody binding was eventually determined using horseradish peroxidase (HRP)-conjugated secondary antibodies (DAKO) and visu-

alised with the POD chemoluminescence kit (Roche Diagnostics). WB membranes were imaged with Molecular Imager Gel Doc XR System (Biorad). Equal protein loading was checked for with the Bradford total protein assay, Ponceau S staining of the blots and ß-actin immunobloting.

Quantitative real-time PCR for p21 (CDKN1A). Total RNA was isolated using the RNeasy Midi Kit according to the manufacturer's instructions (Qiagen). cDNA was synthesized from total RNA as described by the manufacturer's protocol (Life Technologies) using oligo dT primers and M-MLV transcriptase. Quantitative real-time PCR was performed using SYBR Green qPCR SuperMix (Invitrogen). P21 mRNA levels were normalized to the level of GAPDH in the same sample. Results of at least 3 experiments in duplicate are expressed as mean ± SD. Used primers: GAPDH For: CAC CAC CAR GGA GAA CGC TGG, GAPDH Rev: CCA AAG TTG TCA TGG ATG ACC, P21 For: CCT GTC ACT GTC TTG TAC CCT, P21 Rev: GCG TTT GGA GTG GTA GAA ATCT.

Immunofluorescence. Cells were fixed with 4% paraformaldehyde and blocked with 0.1% Triton X-100, 1% BSA and 1% NGS in PBS. Followed by immunostaining with the corresponding antibodies and counterstained with Alexa-Fluor goat secondary antibodies (Molecular Probes, Invitrogen). Finally, cells were stained with Hoechst 33258 (Molecular Probes, Invitrogen) and mounted with Vectashield (Vector Laboratories). Quantimet 600S system (Leica Microsystems) was used for analysis. The data were exported as bmp files and processed using Corel Photo-paint X4 (Corel Corperation).

Caspase 3 activity. Caspase-3 was assayed according to the manufacturer's instructions using DEVD-MCA (Zebra Bioscience BV). Fluorescence was monitored in an FL600 Fluorimeter Bio-tek plate reader (Beun de Ronde).

Tera-p21-Δ-NLS stably transduced cell line. pMSCV-p21-Δ–NLS, pMSCV-IGFP and pCL-Ampho were kindly provided by Dr. J.J. Schuringa & Dr. H Schepers (Hematology Research, University Medical Center Groningen, the Netherlands). For viral production, 293T cells were transfected with pCL-Ampho and MSCV-IGFP, either with or without p21-Δ–NLS. GFP positive Tera cells were sorted on a fluorescent-activated cell sorter (MoFlow, Cytomation).

RNA interference & miRNA antisense & Transfections. Specific siRNA and negative control (scrambled) were purchased from Eurogentec. Synthetic anti-miRNA were purchased from IDT and pre-miR-17-5p from Ambion. EC cells were transfected in 6 well plates with 5 µl of 20 µM siRNA duplex or miRNA antisense using Oligofectamine reagent according to the manufacturer's instructions (Invitrogen). After 24h, cells were treated with cisplatin. 24h after the treatment cells were harvested for protein isolation. Alternatively, in order to perform an apoptosis assay, at 24h after transfection, cell were harvested and plated in 96-well plate. The day after, cells were treated with cisplatin. For the luciferase reporter assay Tera cells were transfected with psiCHECK2-p21-3'UTR using Fugene, lysates were made and assay was performed as described previously (26). p21-I: 5'-CUU CGA CUU UGU CAC CGA GdTdT (sense), 5'-CUU ACG CUG AGU ACU UCG AdTdT (anti-sense); P21-II: 5'-GAC CAU GUG GAC CUG UCA CTdT (sense), 5'-GUG ACA GGU CCA CAU GGU CdTdT (antisense); CDK2: 5'-GCC AGA AAC AAG UUG ACG GGA dTdT (sense), 5'-UCC CGU CAA CUU GUU UCU GGC dTdT (anti-sense); CDK2 II: 5'-GGU GGU GGC GCU UAA GAA AdTdT (sense), 5'-UUU CUU AAG CGC CAC CAC CdTdT (anti-sense); Akt1: 5'-GGA GGG UUG GCU GCA CAA AdTdT (sense), 5'-UUU GUG CAG CCA ACC CUC CdTdT (anti-sense); Akt2: 5'-CUU CUC CGU AGC AGA AUG CdTdT (sense), 5'-GCA UUC UGC UAC GGA GAA GdTdT (anti-sense); Akt3: 5'-CUG GAG GCC AAG AUA CUU CdTdT (sense), 5'-GAA GUA UCU UGG CCU CCA GdTdT (anti-sense); Oct4 I: 5'-CAU GUG UAA GCU GCG GCC CdTdT (sense), 5'-GGG CCG CAG CUU ACA CAU GdTdT (anti-sense), Oct4 II: 5'-AGC AGC UUG GGC UCG AGA AdTdT (sense), 5'-UUC UCG AGC CCA AGC UGC UdTdT (anti-sense).

Immunoprecipitation. Cells (107) were harvested, washed with ice-cold PBS, and lysed in 500 µl lysis buffer (20 mM Tris HCl pH 7.6, 150 mM NaCl, 0.2% NP-40, protease inhibitor COMPLETE®, 1 mM PMSF, 1mM NaF and 1 mM DTT). Lysates were clarified and protein concentration was equalized with Bradford, and incubated for 16h with a mixture of agarose conjugated anti-p21 (F5 & C19, Santa Cruz). Immunocomplexes were washed 5 times and eluted with 0.5 M Glycine/HCl pH 2.4, mixed 1:1 with standard 2x Western Blot sample buffer and examined

by Western blot analysis as described above.

Facs analysis p-p21 (Thr145). For FACS analysis of the levels of phosphorylated p21 EC cells were seeded in 6 wells plates and 24h after cisplatin or gamma-irradiation treatment the cells were collected, washed with PBS and fixed with Methanol/Aceton for 20 min at room temperature. Fixed cells were washed 2 times with 1% BSA in PBS followed by immunostaining with p-p21 antibody in 1% BSA in PBS and counterstained with Alexa-Fluor secondary antibody. Fluorescence intensity was detected with the FACS-Calibur (Becton Dickinson).

Patients, immunohistochemistry & miRNA in situ hybridization. General patient characteristics and histopathology are summarized in Supplemental Table 1. The TC patients with chemo-sensitive disease have been reported before (27). From the patients with TC diagnosed and treated at our institution between 1985 and 2007 with refractory disease (defined as patients not achieving a response on initial treatment or renewed elevation of tumor marker levels within 4 weeks after completion of chemotherapy), patients with sufficient EC component in their tumor samples (n=7) were selected. TC specimens were used to represent all histological subtypes of the primary tumor (Embryonal Carcinoma = EC: Yolk Sac Tumour = YS; Choriocarcinoma = ChC; Mature Teratoma/Immature Teratoma =T; Seminoma = S) and each of the three different prognosis groups according to the IGCCCG classification (28). The studies were approved by the medical ethical committee of University Medical Center Groningen, the Netherlands, and all patients gave informed consent prior to study entry. For each patient, representative paraffin embedded tumor material was collected and serial 3-μm sections were cut.

For immunohistochemistry sections were deparaffinized in xylene and rehydrated in alcohol; antigen retrieval was performed followed by blocking of endogenous peroxidase (30 min, 3% H2O2). Subsequently, slides were incubated for 1h with the primary antibodies: Oct4 (C20, Santa Cruz), p21 (EA10, Oncogene), p-p21 (Thr145, Santa Cruz) and Ki-67 (MIB-1, Dako) and counterstained with HRP-conjugated secondary antibodies (DAKO). DAB was used as chromagen to visualize peroxidase activity. Counterstaining was performed with hematoxylin. Immunoglobulin class-matched control sera were used as negative controls. Normal colon and normal skin served as a positive control for p21.

miRNA ISH was performed as reported previously (29). For both ISH and IHC slides were scanned using ScanScope CS System (Aperio) and pictures were taken with ImageScope software package (Aperio).

RNA isolation & TaqMan miRNA quantitative PCR. Total RNA was isolated and miRNA-106b family expression was measured as previously described (26). The miRNA expression was normalized to RNU48 expression resulting in a ΔCt from which the $2^{-\Delta Ct}$ value was derived and depicted.

Statistical Analysis. Results of at least 3 experiments are expressed as mean ± SD. Student's unpaired t test was used to compare values of test and control samples. Differences were considered significant when at least $p < 0.05$. Fisher exact $\chi 2$ test was used for categorical patient variables. Differences were considered significant when at least, with 2 sided $p < 0.05$.

Results

High cytoplasmic p21 expression is associated with cisplatin-resistance in EC cells. A panel of cisplatin-sensitive and -resistant EC cell lines was used in this study to compare cisplatin responses (Figure 1A & Supplemental Figure 1A) with the levels of (induced) p21 (Figure 1B-C). Resistance to cisplatin was predominantly associated with high p21 expression levels, since the intrinsically resistant EC cell lines Scha and 2102EP showed higher basal and cisplatin-induced p21 and *CDKN1A* levels compared to the cisplatin-sensitive EC cell lines Tera and 833KE (Figure 1A-C). Low levels of p21 were not associated with cisplatin response in Tera-CP, a subline with acquired cisplatin-resistance due to p21 unrelated mechanisms (23).

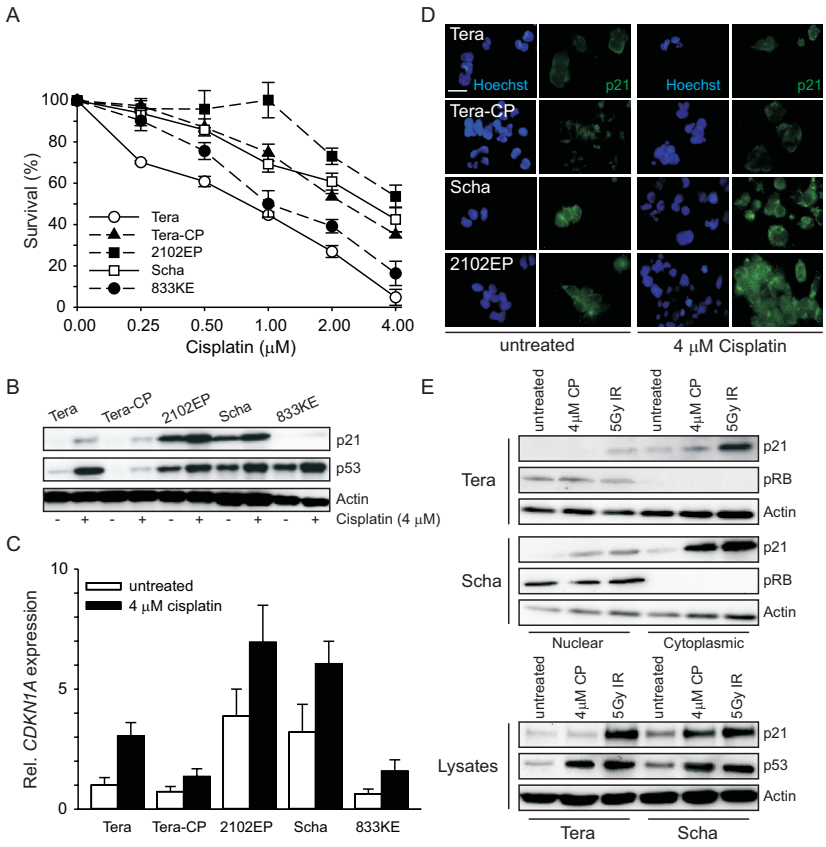

Figure 1. High cytoplasmic p21 expression is associated with cisplatin-resistance in EC cell lines. (A) Survival of EC cells after 96h of continuous cisplatin treatment as indicated. (B) Difference in p21 and p53 levels of the EC cell lines treated and untreated with cisplatin for 24h as indicated. A representative example of three independent experiments is shown. (C) Difference in *CDKN1A* expression levels of the EC cell lines treated and untreated. Note that the intrinsic cisplatin-resistant EC cell lines (2102EP and Scha) have higher basal and cisplatin-induced p21/*CDKN1A* levels compared to cisplatin-sensitive EC cell lines (Tera 833KE). (D) p21 is localized in the cytoplasm in EC cells when untreated and after 24h of cisplatin treatment. Scale bar, 30 μm. (E) 24h after cisplatin (CP) treatment or gamma-irradiation (IR), nuclear and cytoplasmic proteins were isolated and analyzed by WB for expression of p21 using retinoblastoma protein (pRB) as nuclear control whereas β-actin is shown as a loading control. WB of total lysates show that, despite an almost similar p53 increase, gamma-irradiation in contrast to cisplatin treatment, strongly induced accumulation of p21 both in cisplatin-resistant Scha and cisplatin-sensitive Tera cells. Representative examples of three independent experiments are shown.

The subcellular localization of p21 was investigated with immuno-fluorescence microscopy (IF) and Western blot analysis (WB) using fractionated protein samples. We stained the EC cell lines Tera, Tera-CP, 833KE, Scha and 2102EP for basal levels of p21 and p21 levels after treatment with cisplatin (or gamma-irradiation). All untreated EC cell lines showed a predominant cytoplasmic localization of p21, albeit with large differences in p21 intensity (Figure 1D-E). Cytoplasmic localization was not affected by cisplatin treatment (Figure 1D-E & Supplemental Fig 1B). Gamma-irradiation was used as positive control for

p21 induction in EC cells (7). Despite almost a similar p53 increase, gamma-irradiation, in contrast to cisplatin treatment, strongly induced accumulation of p21 in the cytoplasm in both cisplatin-resistant Scha and cisplatin-sensitive Tera cells (7)(Figure 1E & Supplemental Figure 1C). We next hypothesized that high levels of cytoplasmic p21 have a causal role in cisplatin-resistance in EC cell lines.

Cytoplasmic p21 protects EC cells against cisplatin- induced apoptosis. To investigate the importance of high levels of cytoplasmic p21 both cisplatin-sensitive and cisplatin-resistant EC cell lines were depleted of p21, using siRNA. Downregulation of p21 led to an increase in apoptosis upon cisplatin treatment in the intrinsically resistant cell lines Scha and 2102EP as compared to cells transfected with scrambled siRNA (Figure 2A). The loss of p21 in Scha and 2102EP resulted in enhanced PARP cleavage and higher caspase 3 activation compared to control cells following cisplatin treatment (Figure 2B-C). In the cisplatin-sensitive 833KE and Tera cells and the acquired cisplatin-resistance Tera-CP cells, all expressing low endogenous cytoplasmic p21 levels, no significant effect of p21 downregulation on apoptosis levels was observed (Supplemental Figure 2A-C).

Next, we stably transduced the cisplatin-sensitive cell line Tera with a retroviral construct containing both p21-Δ-NLS and Green Fluorescent Protein (GFP), or GFP only (Tera-mock) to study more extensively whether enhanced levels of cytoplasmic localized p21 is involved in suppressing apoptosis. The p21-Δ–NLS protein lacks its bipartite nuclear localization signal and is therefore maintained in the cytoplasm (Supplemental Figure 2D). Treatment with cisplatin resulted in less apoptosis and reduced caspase 3 activity in Tera-p21-Δ–NLS compared to Tera-mock (Supplemental Figure 2E-F). We downregulated p21-Δ–NLS in Tera-p21-Δ-NLS cells using p21 siRNA, and we showed that these cells became as sensitive as Tera-mock cells to cisplatin. No effect of p21 siRNA on cisplatin-induced apoptosis was observed in Tera-mock cells (Figure 3A-B & Supplemental Figure 2G-H).

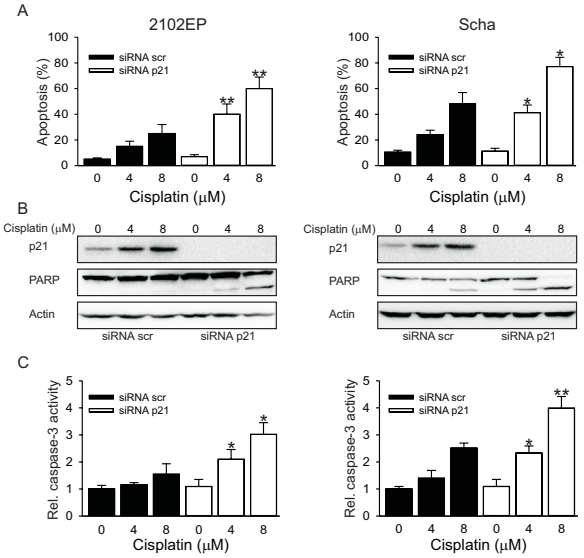

Figure 2. Increased cisplatin sensitivity after p21 downregulation in intrinsically resistant EC cell lines. (A) Downregulation of p21 sensitizes intrinsically resistant EC cells for cisplatin-induced apoptosis. Cells were treated with scrambled siRNA (siRNA scr) or with p21 siRNA (siRNA p21) for 24h. After cisplatin treatment for 24h apoptosis induction was analyzed by fluorescence microscopy on acridine orange stained cells for 2102EP and Scha. (B) Successful downregulation of p21, using siRNA against p21, and enhanced cleavage of PARP in cisplatin treated 2102EP and Scha, a representative example of three independent experiments is shown. (C) Increased caspase 3 activity, after treatment with siRNA against p21, in cisplatin treated 2102EP and Scha. *p < 0.05; **p < 0.01 compared to matching siRNA scrambled (scr) control.

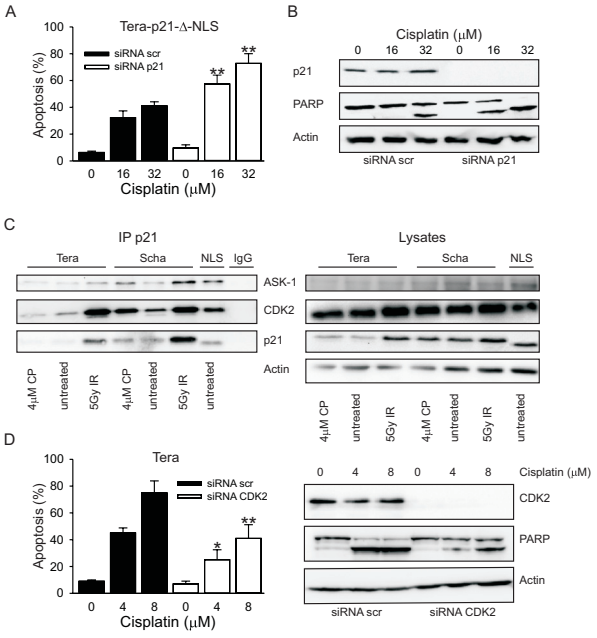

Figure 3. Over-expression of cytoplasmic p21 protects against cisplatin induced apoptosis via complex formation of p21 with CDK2 and ASK1. (A) p21 siRNA mediated downregulation of p21-Δ–NLS in Tera-p21-Δ-NLS sensitizes for cisplatin induced apoptosis. (B) Western blot analysis showing downregulation of p21-Δ–NLS and enhanced cisplatin-induced cleavage of PARP in Tera-p21-Δ–NLS after treatment with p21 siRNA (C) EC cells were harvested 24h after gamma-irradiation (IR) or cisplatin (CP) treatment. Cell lysates were subjected to p21 immunoprecipitation (IP). Immunoblotting was performed using anti-p21, anti-CDK2, and anti-ASK1 antibodies. In Scha and Tera-p21-Δ–NLS higher amounts of p21 are precipitated and more CDK2 and ASK1 are co-precipitated compared to Tera, whereas in irradiated Tera and Scha almost similar levels of p21, CDK2 and ASK1 are co-precipitated. The data presented are representative of three independent experiments. (D) CDK2 acts pro-apoptotic after 24h cisplatin treatment in Tera. Decreased apoptotic response and increased PARP cleavage after successful downregulation of CDK2. *$p < 0.05$; **$p < 0.01$ compared to matching siRNA scrambled (scr) control.

Cytoplasmic p21 forms a complex with pro-apoptotic CDK2 in cisplatin-resistant EC cells. It has been described that caspase 3 mediated cleavage of p21 activates the proapoptotic role of cyclin dependent kinase 2 (CDK2) (30-32). Therefore, we analyzed the presence of CDK2 in p21 immunoprecipitates. As shown in Figure 3C, in Tera, Tera-p21-Δ–NLS and Scha CDK2 co-precipitated with p21, indicating that CDK2 is in complex with cytoplasmic p21 in untreated cells and in cells treated with cisplatin or gamma-irradiation (Figure 3C). In addition, we stained for the presence of ASK1 since cytoplasmic p21 can bind to apoptosis signaling kinase 1 (ASK1) thereby inhibiting ASK1-mediated apoptosis via the intrinsic mitochondrial apoptotic pathway (10-13). In fact, more CDK2 and ASK1 was co-precipitated with p21 in untreated and cisplatin-treated Scha and Tera-p21-Δ–NLS, which have higher p21 levels compared to Tera. In irradiated Tera and Scha, expressing equal p21 levels, high levels of p21 were precipitated leading to co-precipitation of almost similar levels of CDK2 and ASK1 (Figure 3C). We further established the important proapoptotic role of CDK2 by downregulating CDK2 with siRNA in cisplatin-sensitive cell lines, which led to a decrease in cisplatin-induced apoptosis and PARP cleavage compared to scrambled siRNA in Tera and 833KE (Figure 3D & Supplemental Figure 3A). Next, we investigated the pro-apoptotic role of CDK2 in the high p21 expressing 2102EP and Scha. Therefore, we sensitized these cells to cisplatin by suppression of p21 and, additionally, transfected these cells with CDK2 siRNA. The observed increase in apoptosis in p21 suppressed 2102EP and Scha cells upon cisplatin treatment could be dramatically reduced by co-transfection with CDK2 siRNA (Supplemental Figure 3B).

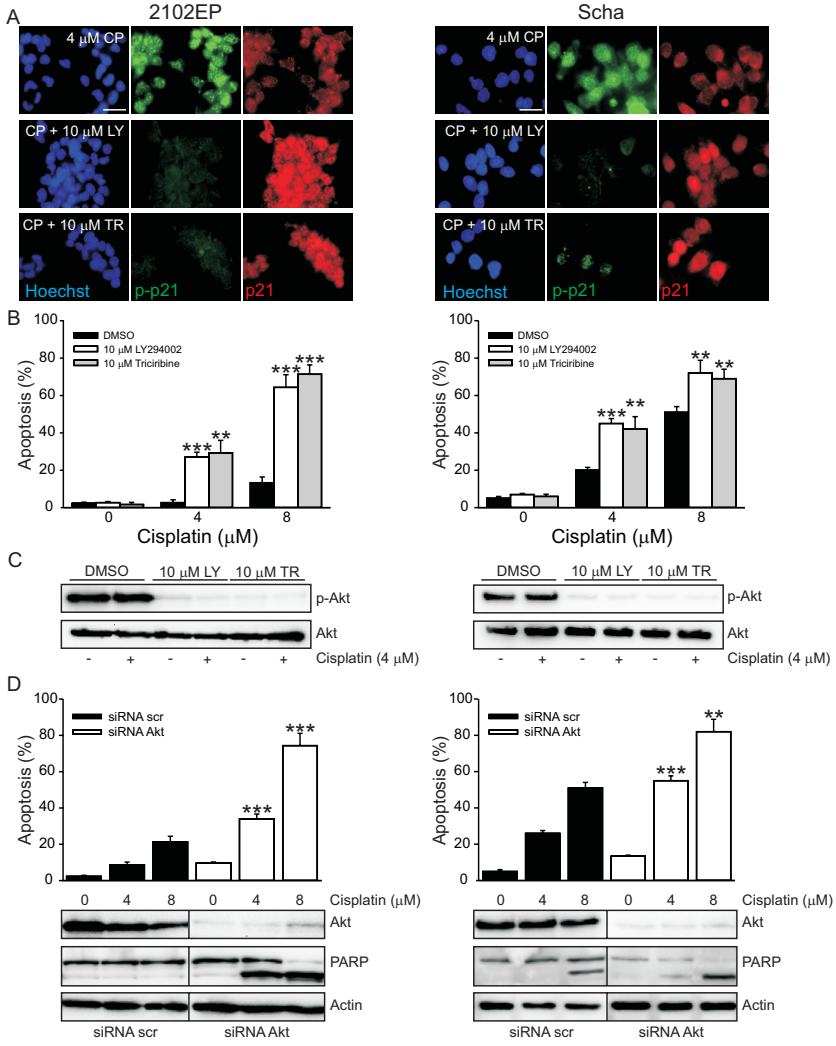

Figure 4. Dephosporylation of p-p21, nuclear localization of p21 and sensitization for cisplatin after combined treatment with LY294002 or Triciribine. (A-C) 24h after cisplatin (CP) treatment in combination with either 10 μM LY294002 (LY) or 10 μM Triciribine (TR) decreased phosphorylated levels of p21(Thr145), whereas p21 is more pronounced localized in the nucleus of 2102EP and Scha (Scale bar, 30 μm) (A) and dephosporylation of p-Akt occurred after treatment with LY294002 or Triciribine in Scha and 2102EP (B). This treatment led to increased apoptosis induction in 2102EP and Scha (C). **p < 0.01; ***p < 0.001 compared to matching DMSO control. (D) siRNA against Akt1-3 sensitizes intrinsically resistant EC cell lines for cisplatin. Akt1-3 downregulation increased apoptosis induction and enhanced PARP cleavage after cisplatin treatment in 2102EP and Scha. **p < 0.01; ***p < 0.001 compared to matching siRNA scrambled (scr) control.

Interfering in p-Akt mediated cytoplasmic localization of p21 sensitizes cisplatin-resistant EC cells. The cytoplasmic localization of p21 can be caused by phosphorylation of Thr-145 located in the NLS of p21 (11,33). Upon phosphorylation of the NLS domain by p-Akt phosphorylated p21 (p-p21) becomes more stable and is dynamically shuttling between

the nucleus and the cytoplasm (11,33-34). In untreated and cisplatin-treated EC cells, p-p21 was detectable with WB (Supplemental Figure 4A). IF indicated that p-p21 was also localized in the cytoplasm (Figure 4A). In order to find out if phosphorylation of Thr-145 could occur via p-Akt, we stained the EC cells for p-Akt and its natural inhibitor PTEN. In Tera, Scha and 2102EP p-Akt is present (Figure 4B & Supplemental Figure 4B), whereas PTEN is not expressed in these cells (data not shown)(35).

Treatment with the PI3K inhibitor LY294002 or with the specific Akt inhibitor Triciribine (currently in phase I trial) resulted in dephosphorylation of Akt in Tera, Scha and 2102EP and concomitant dephosphorylation of p21 (Thr-145), which is consistent with a more pronounced nuclear localization of p21 (Figure 4A,B & Supplemental Figure 4B-E). In the cisplatin-sensitive cell line Tera either LY294002 or Triciribine did not affect cisplatin sensitivity (Supplemental Figure 5A), though shuttling of the weakly detectable cytoplasmic p21 to the nucleus was observed (Supplemental Figure 4C,D). Interestingly, a strong sensitization to cisplatin was observed in Scha and 2102EP with the combined treatment for 24h with cisplatin and either LY294002 or Triciribine (Figure 4C & Supplemental Figure 5A). Shuttling of p21 to the nucleus following LY294002 treatment resulted in a loss of CDK2 in complex with p21 in Scha cells (Supplemental Figure 5B). Release of CDK2 was instrumental in the enhanced induction of apoptosis, since the sensitization to cisplatin combined with LY294002 in 2102EP and Scha cells was almost completely blocked following suppression of CDK2 (Supplemental Figure 5C). Downregulation of Akt using siRNA targeting Akt1-3 reduced the overall levels of p-p21 and resulted in a more pronounced nuclear localization of p21 (Supplemental Figure 5D). In addition, Akt1-3 suppression sensitized 2102EP and Scha for cisplatin-induced apoptosis (Figure 4D).

Enhanced cytoplasmic p21 positivity and low levels of apoptosis were found in Tera and Scha 24hrs after gamma-irradiation (Figure 1E & Supplemental Figure 4D). The combined treatment of gamma-irradiation with either LY294002 or Triciribine for 24h resulted in less p-p21, predominantly nuclear localization of p21, and augmented apoptosis in both Tera and Scha (Supplemental Figure 4B,D & 5E). In Tera-p21-Δ–NLS, lacking the phosphorylation site Thr145, treatment with the PI3K/Akt inhibitors, however, had no effect on the cytoplasmic localization of p21-Δ–NLS (Supplemental Figure 4C) and treatment with the inhibitors did not lead to sensitization to cisplatin (data not shown). These results show that the sensitizing effect of PI3K/Akt inhibitors on cisplatin-induced apoptosis is mediated via dynamic changes in p21 localization.

Cytoplasmic expression of p21 and p-p21 in the EC component of chemo-refractory TC patients. Next, we investigated the p21 and p-p21 (Thr145) expression patterns in EC containing TC tissue from 23 patients with metastatic disease, who were cured and considered sensitive to cisplatin containing chemotherapy and from 7 patients with metastatic disease not cured and considered refractory to cisplatin containing chemotherapy. Histological analysis after H&E staining was used to distinguish the various TC components. In addition, Oct4 (also known as POU5F1 and Oct3/4) immunohistochemistry (IHC) was used as marker for embryonal pluripotency (16). Mature teratoma showed an intense p21 and p-p21 staining, whereas no staining of Oct4 was noticed (Supplemental Figure 6A). EC components from TC patients, sensitive to cisplatin containing chemotherapy, were negative for p21 in 23 (100%) and negative for p-p21 in 21 (91%) of the patients (Figure 5A & Supplemental Figure 6A). In contrast, the EC component of refractory TC patients stained positive for p21/p-p21 in 6 out of 7 (86%) patients, including 2 patients with EC as only component (Figure 5B).

Positive staining for p21/p-p21 in the EC component was significantly more often observed in refractory TC patients compared with TC patients sensitive to cisplatin containing chemotherapy ($P < 0.0003$, Fisher's exact test). Moreover, IF showed that the localization of p21/p-p21 in the EC component of refractory patients was mainly cytoplasmic, whereas the localization of p21/p-p21 in teratomas is more nuclear (Figure 5C). In addition, presence of Ki-67 staining indicated proliferation instead of cell cycle arrest in abundantly p21/p-p21-positive EC (Supplemental Figure 6B). Taken together, our results show that cytoplasmic p21 and p-p21 positivity of EC components in TC patients is associated with the response (poor outcome) to cisplatin-containing chemotherapy.

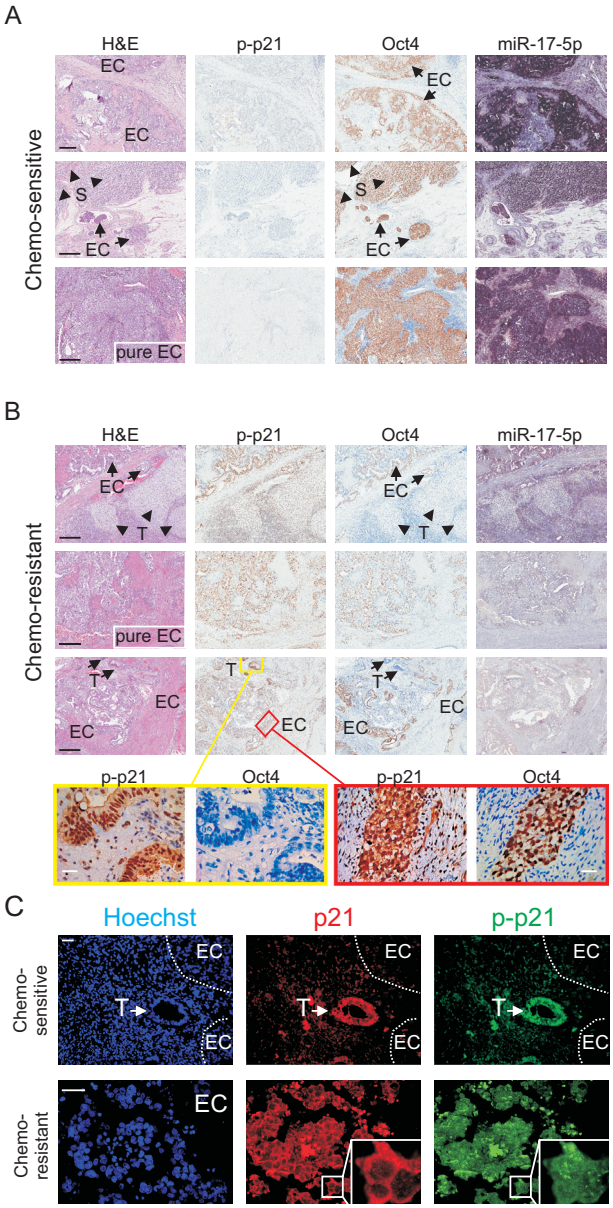

Figure 5. Cytoplasmic p21 expression in embryonal carcinoma of non-responding TC patients. (A) Embryonal carcinoma (EC) or seminoma (S) components of chemo-sensitive patients stain negative for p21 (IHC) and intensively positive for Oct4 (IHC) and miR-17-5p (ISH). Representative examples of staining are shown. Scale bar, 300 µm. (B) The embryonal carcinoma component (EC) of patients refractory to cisplatin-containing chemotherapy, in contrast to responders, stains in the majority of cases positive for p21 and less positive for Oct4 and miR-17-5p. All teratoma component (T) stains positive for p21/p-p21 and negative for Oct4 and miR-17-5p. Representative examples of staining are shown. Scale bar, 300 µm. Pictures of the panels with higher magnification were made with Leica DM3000 (Leica Microsystems). Scale bar, 30 µm. (C) Immunofluorescent staining showing that p21 is nuclear localized in mature teratoma (T) and negative in chemo-sensitive EC patients, whereas p21 is cytoplasmic localized in the EC component of refractory patients. Representative example of stainings is shown. Scale bar, 30 µm. Insets represent a selected area of the original image as indicated, 4 times digitally magnified.

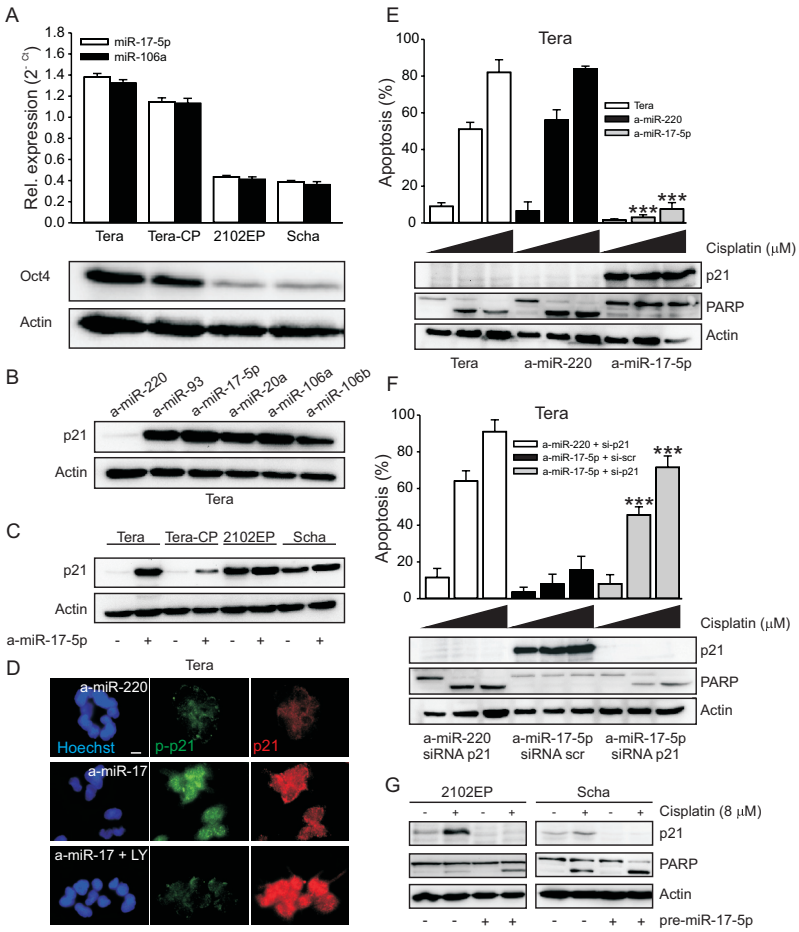

Figure 6. Positivity for p21 and p-p21 protein is inversely associated with the expression of the miR-106b seed family in EC. (A) Quantitative RT-PCR on miR-106b seed family expression in EC cell lines. Note that the intrinsic cisplatin-resistant EC cell lines (2102EP and Scha) have lower levels of the miR-106b seed family and lower Oct4 expression compared to cisplatin-sensitive EC cell lines (Tera and 833KE). All reactions were run in triplicate. The miRNA expression was normalized to RNU48 expression resulting in a ΔCt from which the $2^{-\Delta Ct}$ value was derived and depicted. (B) Enhanced p21 levels in Tera 24h after treatment with synthetic anti-miR-106b seed family members. Synthetic anti-miR-220 was used as negative control, since miR-220 expression was not detectable in TC (38). (C) Differences in p21 enhancement in EC cells 24h after synthetic anti miR-17-5p treatment. (D) Levels of cytoplasmic localized p21 were enhanced after treatment with synthetic anti-miR-17-5p (a-miR-17), compared to control (a-miR-220). Treatment with 10 μM LY294002 (LY) decreased phosphorylated levels of p21(Thr145) and p21 became more localized in the nucleus of synthetic anti-miR-17-5p transfected Tera cells (Scale bar, 30 μm) (E) Synthetic anti-miR-17-5p strongly up-regulates p21 and reduces cisplatin induced apoptosis and reduces PARP cleavage in Tera. Cells were treated with 0, 4 and 8 μM cisplatin. ***$p < 0.001$ compared to matching anti-miR-220 control. (F) Reduced apoptosis induction and reduced PARP cleavage depends on upregulation of p21 by synthetic anti-miR-17-5p, as shown by co-transfection with siRNA against p21. Cells were treated with 0, 4 and 8 μM cisplatin. ***$p < 0.001$ compared to matching anti-miR-17-5p + scrambled siRNA. (G) Lower expression levels of p21 and enhanced PARP cleavage 24 hours after cisplatin treatment in pre-miR-17-5p transfected Scha and 2102EP cells.

Reduced levels of Oct4 and miR-106b family members causes high cytoplasmic p21 expression in cisplatin-resistant EC cells. Involvement of miRNAs belonging to the miR-106b seed family in regulating p21/CDKN1A and cell cycle control has previously been demonstrated (36-37), Therefore, we examined the expression levels of the miR-106b seed family in the EC cell lines. The intrinsically resistant Scha and 2102EP cell lines, with high p21 levels, showed lower levels of the miR-106b seed family compared to the cisplatin-sensitive Tera and 833KE cell lines (Figure 6A & Supplemental Figure 6C). Subsequently, we used synthetic anti-miRNA to specifically inhibit the miR-106b seed family and to demonstrate its relation with localization and expression level of p21 in EC cell lines. In Tera cells, transfection with synthetic anti-miRNA against miR-17-5p, miR-20a, miR-93, miR-106a, or either miR-106b resulted in a massive increase in cytoplasmic p21 levels compared to control (Figure 6B,D). Although treatment with synthetic anti-miR-17-5p resulted in enhanced p21 levels in Tera, Tera-CP, Scha and 2102EP, the strongest enhancement of p21 was found in Tera and Tera-CP (Figure 6C), which may at least in part be explained by the higher miR-17-5p levels in these cell lines (Figure 6A) (38). The strongly enhanced cytoplasmic p21 levels in Tera following synthetic anti-miR-17-5p treatment completely blocked cisplatin-induced apoptosis (Figure 6D,E). Suppression of cytoplasmic p21 with siRNA or shutteling of p21 to the nucleus with LY294002 resensitized synthetic anti-miR-17-5p treated Tera cells to cisplatin (Figure 6F & Supplemental Figure 6D). Overexpression of pre-miR-17-5p resulted in lower p21 levels and an increase in cisplatin-induced apoptosis in Scha and 2102EP cells (Figure 6G), demonstrating the important causal relation between the presence of miR-17-5p, the reduced p21 expression and high cisplatin sensitivity.

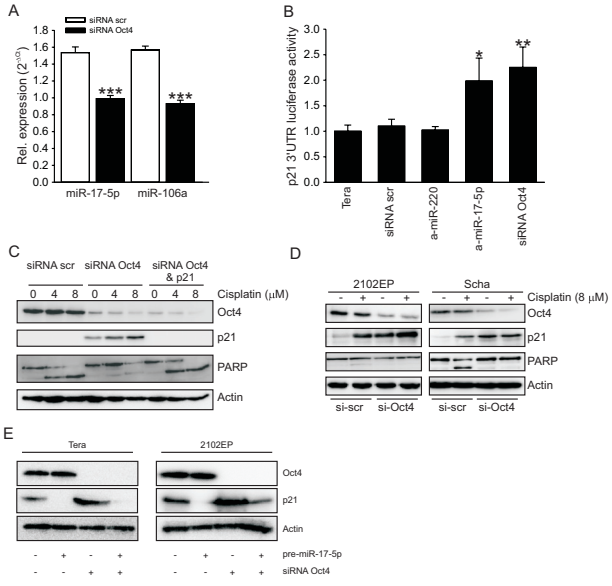

Figure 7. Oct4 regulates the expression level of the miR-106b seed family in EC. (A) Quantitative RT-PCR on miR-106b seed family expression in EC cell lines, showing less expression of the miR-106b seed family in Oct4 suppressed cells, after 48h of treatment with siRNA against Oct4, compared to control. ***$p < 0.001$ compared to control. (B) Reporter assay showing increased p21-3'UTR dependent luciferase translation in cells 48h after cotransfection of psiCHECK2-p21-3'UTR with either anti-miR-17-5p or Oct4 siRNA. Bars indicate Firefly luciferase activity normalized to Renilla luciferase activity. *$p < 0.05$; **$p < 0.01$. (C,D) Oct4 suppression leads to upregulation of p21 and concomitantly resistance to cisplatin in Tera (C), 2102EP and Scha cells (D), while downregulation of p21 with siRNA resensitized Oct4 suppressed Tera cells for cisplatin (C). (E) Oct4 suppression leads to upregulation of p21 in Tera and 2102EP cells. Pre-miR-17-5p transfection abolished p21 expression in Tera and 2102EP cells and diminished p21 upregulation after Oct4 suppression to less than basal p21 expression levels.

We performed in situ hybridization (ISH) for miR-106a and miR-17-5p on paraffin-embedded formalin-fixed tumor tissue from patients with multi-component (primary) TC, to define in-vivo the relation between p21/p-p21 expression and the presence of miR-106b seed family members in the EC component. A strong positive staining for miR-17-5p, miR-106a and Oct4 but not for p21/p-p21 was found in the EC components of chemo-sensitive patients. In contrast, mature teratomas showed no staining of miR-17-5p, miR-106a and Oct4, while stained intensively positive for p21/p-p21 (Figure 5A & Supplemental Figure 6A). On the contrary, in chemo-refractory TC patients almost no miR-17-5p staining was observed in EC components, while p21/p-p21 was clearly detectable. Moreover, the staining intensity of Oct4 in the EC component is less pronounced in refractory patients compared to chemotherapy-sensitive patients (Figure 5B). Additionally, the expression levels of both Oct4 and miR-106b seed family members are lower in the intrinsically resistant cell lines Scha and 2102EP compared to the cisplatin-sensitive cell line Tera (Figure 6A). These results suggest a relation between the reduced expression of Oct4 and the miR-106b seed family member in refractory ECs. In murine embryonic stem cells, Oct4 has been implied in controlling expression of several miRNA families, including miR-17~92 and miR-106a~363 cluster of the miR-106b seed family (39). We downregulated Oct4 in Tera cells with siRNA, in order to establish the direct relation between Oct4, miR-106b and p21 expression in ECs. Suppression of Oct4 lowered the expression level of miR-106a, miR-17-5p and miR-20a compared to control Tera cells (Figure 7A & Supplemental Figure 6E), and resulted in enhanced p21-3'UTR dependent luciferase translation (Figure 7B) and robust induction of p21 (Figure 7C & Supplemental Figure 6F). A similar enhancement of p21-3'UTR dependent luciferase translation was observed following transfection of Tera cells with synthetic anti-miR-17-5p (Figure 7B). Furthermore, Oct4 suppression in Tera, Scha and 2102EP resulted in an upregulation of p21 and a concomitant resistance to cisplatin, which could be reverted by p21 downregulation (Figure 7C-D). Sustained miR-17-5p expression using pre-miR-17-5p precluded the induction of p21 protein even in the context of Oct4 knockdown in Tera and 2102EP cells (Figure 7E). Some p21 expression, however, was still observed in 2102EP cells, albeit at a lower level compared to control 2102EP cells, indicating that not all effects of Oct4 on p21 expression are mediated by the miR-106b seed family. Overall these results reveal an important role of Oct4 in regulating p21 expression via the miR-106b seed family in EC.

Discussion

The functionality of many proteins is thought to be related to their intracellular localization. Nuclear localized p21 is known to control the cell cycle and DNA replication, whereas cytoplasmic p21 has been implicated in the inhibition of apoptosis. Here, we have demonstrated that high levels of cytoplasmic localized p21 protect embryonal carcinoma (EC) cells against cisplatin-induced apoptosis. Furthermore, we dissected the pathway involved in the regulation of p21 expression levels, i.e. OCT4 and the miR-106b family, and identified the key protein causing the cytoplasmic localization of p21. Our results indicate that p-Akt mediated p21 phosphorylation is essential for p21 localization in the cytoplasm. Inhibition of p-Akt retained p21 in the nucleus resulting in less p21 complex formation with CDK2 and sensitized EC cells to cisplatin-induced apoptosis. In cisplatin-sensitive EC cell lines and EC components of chemo-sensitive TC patients, p21 is not commonly expressed, which is related to the expression levels of miR-106b seed family members and Oct4. On the contrary, in cisplatin-resistant EC cell lines and EC components of refractory TC patients, indicating

clinical cisplatin-resistance, cytoplasmic p21 expression was clearly detectable and related to reduced Oct4 and miR-106b expression. Therefore, we conclude that high levels of cytoplasmic localized p21 are important determinants of resistance to cisplatin-based chemotherapy in EC (Figure 8).

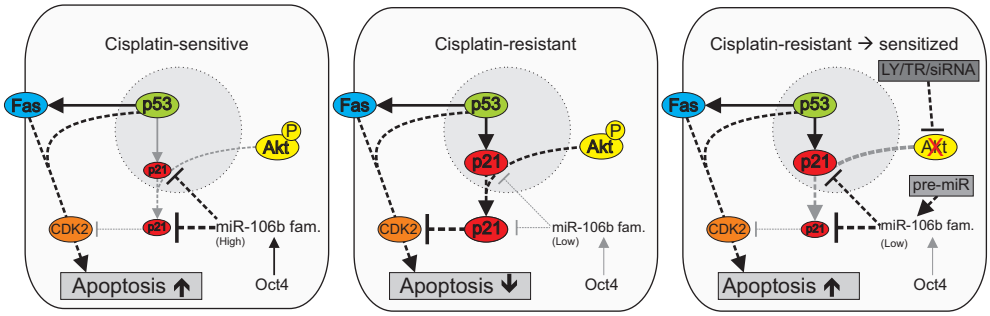

Figure 8. Proposed simplified model showing the mechanisms through which cytoplasmic p21 can inhibit cisplatin-induced apoptosis in EC cells. Cisplatin-induced DNA damage activates p53, which in turn transcribes *CDKN1A* (p21), and activates the Fas apoptosis pathway and the mitochondrial apoptosis pathway. Activated Akt is important for dynamic shuttling of p21 from the nucleus towards the cytoplasm, where p21 can block apoptosis. miR-106b seed family members (miR-106b fam.) are involved in regulating p21 expression levels. Oct4 regulates the expression levels of the miR-106b seed family members. Cisplatin sensitive cells are characterized with high levels of Oct4 and miR-106b family members and as a consequence low amounts of cytoplasmic p21, resulting in cisplatin-induced apoptosis. Cisplatin resistant cells have lower levels of Oct4 and miR-106b family members and high amounts of cytoplasmic p21, resulting in CDK2 inhibition and concomitantly moderate levels of cisplatin-induced apoptosis. Interestingly, deactivation of Akt with LY294002, Triciribine or siRNA against Akt sensitized cisplatin resistant cells. Deactivation of Akt leads to nuclear localization of p21, which in turn is no longer capable of blocking cisplatin-induced apoptosis. Overexpression of pre-miR-17-5p in cisplatin resistant cells resulted in lower p21 levels and an increase in cisplatin-induced apoptosis. Dotted lines indicate interaction, whereas solid lines indicate (p53-induced) transcription.

We recently demonstrated that cisplatin induces apoptosis mainly in a Fas-dependent manner in EC cells (20), while enhanced p21 expression protects EC cells against Fas-mediated apoptosis (7). This protection might be mediated via the inhibition of caspase 3 (40), CDK2 and ASK1 by cytoplasmic p21. We have no indication that p21 binds to caspase 3 and inhibits activation in irradiated EC cells (7) or cisplatin treated EC cells (data not shown). In the present paper, we show that endogenous cytoplasmic p21 forms a complex with CDK2 and ASK1 in EC cells. In EC cells, expressing low p21 levels, CDK2 downregulation strongly reduced the cisplatin induced apoptotic response. Moreover, relocalization of p21 to the nucleus using LY294002, which completely abolished p21-CDK2 complexes, as well as suppression of p21 made initially cisplatin-resistant cells sensitive to cisplatin in a CDK2 dependent manner, indicating the importance of cytoplasmic CDK2 as a pro-apoptotic factor in cisplatin treated EC cells. The pro-apoptotic signaling of CDK2 is not clear yet. It has been reported that cytoplasmic CDK2 activity is induced in a caspase-dependent way following activation of the Fas pathway (41) and leads to depolarization of the mitochondrial membrane potential (42) by cytoplasmic CDK2 mediated translocation of Bax to the mitochondria (42-43). Alternatively, functional interaction of CDK2 with FOXO1 after DNA

damage can be an important mechanism to enhance, among others, Fas ligand expression (44). Taken together, these results indicated that the high cytoplasmic p21 levels, in complex with cytoplasmic CDK2, protect against cisplatin-induced apoptosis in EC cells.

In the present study, in-vivo relevance of p21 expression was demonstrated in the EC component of TC patients with refractory disease, being positive for p21, whereas no p21 was detected in the EC component of patients with chemo-sensitive tumors. Lack of p21 staining in the EC component but strongly positive p21 staining in mature teratoma components has been frequently observed (9,14-15,18). Teratomas, however, predominately show a nuclear localization of p21. Moreover, teratomas in contrast to EC express Rb (17,45) suggesting a putative role for p21 in cell cycle control in these non-growing or rarely growing tumors, also known as growing teratoma syndrome (17). Furthermore, teratomas are, possibly via p21-mediated cell cycle arrest, not responsive to treatment with cisplatin (8,15-17). Interestingly, p21 staining in the EC component of refractory patients was mainly localized in the cytoplasm of EC cells. In addition, Ki-67 staining revealed no cell cycle arrest, but proliferation in EC with abundant expression of p21, indicating a similar role in-vivo for high levels of cytoplasmic p21 in the protection against cisplatin-induced apoptosis as we have demonstrated for cisplatin-resistant EC cell lines. Cytoplasmic p21 expression has been related to drug resistance in several other types of cancer (11). Previous studies in non-TC cell types have shown that phosphorylation of Thr-145 in the p21-NLS by p-Akt is important for dynamic shuttling of p-p21 between the nucleus and the cytoplasm (11,33). PTEN can prevent phosphorylation of Akt in EC cells (35). Virtually all ECs (cell lines and TC patients), however, lack PTEN expression (35), which is shown to be important for the development of invasive TCs (35,46). Here, we show that re-localization of p21 to the nucleus by dephosphorylation of p21, using the PI3K inhibitor LY294002, the Akt inhibitor Triciribine or siRNA against Akt, sensitized cells for cisplatin-induced apoptosis. Phosphorylation of Akt might be inhibited by targeting tyrosine kinase membrane receptors like KIT and ERBB2, that have been implied in TC (8,47-52). An even more direct approach to target Akt phosphorylation, besides Triciribine, is the use of PI3K/Akt inhibitors that are currently in clinical development (53). However, the efficacy of the various subclasses of PI3K- or Akt-inhibitors to relocalize p21 and subsequently enhance cisplatin sensitivity in refractory TC patients needs to be further elucidated.

The role of miRNAs in tumorigenesis is attracting increasing attention and miRNAs have actually been implicated in TC/EC carcinogenesis (54-55). The miR-106b seed family is potentially oncogenic (56) and has been implied in embryonal stem cells in their maintenance and control of differentiation (57). ECs share many similarities with embryonic stem cells (16,38) including their miRNA expression profile (38). Interestingly, our results indicate differences in expression levels of p21-3'UTR regulating miR-106b seed family members between intrinsically cisplatin-resistant EC and cisplatin-sensitive EC cell lines. Additionally, we report that high levels of the miR-106b seed family members are causing low levels of (cytoplasmic) p21 in EC cells and consequently sensitivity to cisplatin. In contrast, low levels of the miR-106b seed family members were associated with high levels of (cytoplasmic) p21 in EC cells and EC of refractory TC patients. In murine embryonic stem cells, miR-17~92 and miR-106a~363 clusters of the miR-106b seed family are under control of embryonic stem cell factor Oct4 (39). ECs, like embryonic stem cells, also express a cassette of pluripotency genes (16,38,58), where Oct4 is supposed to be the key pluripotency regulator (58). Here, we show that expression of members of the miR-106b seed family

colocalizes with Oct4 expression in TC. Our results indicate that Oct4 regulates expression of miR-17-5p, miR-106a and miR-20a, belonging to the miR-106b seed family, via the p21-3'UTR. Furthermore, in EC cells, expressing high basal levels of Oct4, over-expression of miR-17-5p completely precluded p21 induction when Oct4 was suppressed. Similar results were obtained with EC cells, expressing lower basal levels of Oct4, however, some p21 expression was still detectable in these cells after combined transfection of Oct4 siRNA and pre-miR-17-5p. This suggests that besides the miR-106b seed family the regulation of p21 expression through Oct4 may be more complex, involving other factors as well. In summary, we provide evidence that Oct4 regulates expression of p21-3'UTR targeting miR-106b seed family members and thus indirectly p21 levels and cisplatin sensitivity in EC cells.

In conclusion, we demonstrate an important mechanism in EC that causes elevated cytoplasmic p21 levels and consequently results in cisplatin-resistance. Moreover, we provide a target to overcome resistance, which may form the basis for the development of novel therapeutic approaches to alleviate cisplatin-resistance in TCs or other cisplatin-resistant types of solid tumors.

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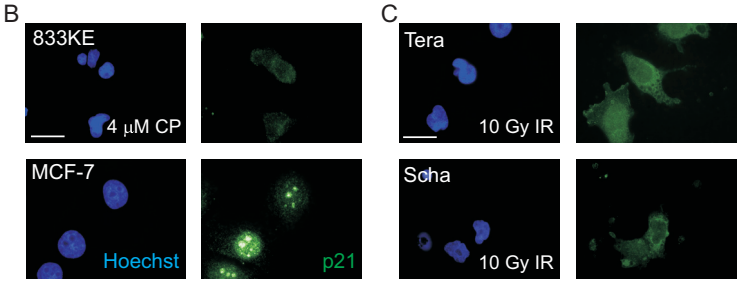
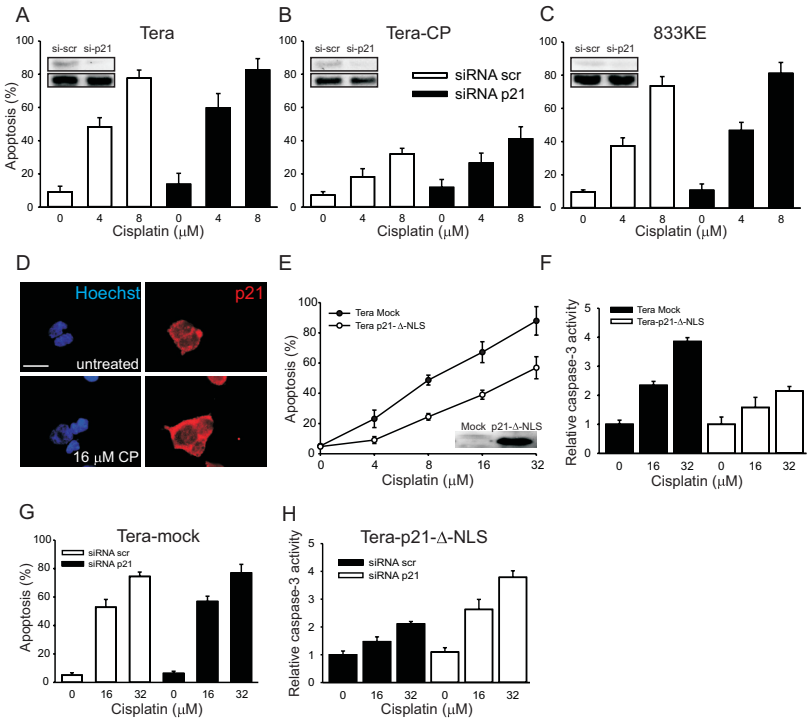
A

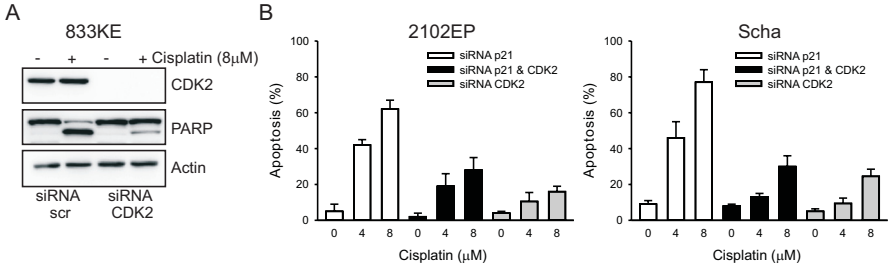
	IC_{50} cisplatin (μM)	Model
Tera	0.69 ± 0.11	Sensitive
833KE	1.04 ± 0.09	Sensitive
Tera -CP	2.10 ± 0.16	Acquired resistant
Scha	2.90 ± 0.84	Intrinsic resistant
2102EP	3.80 ± 0.49	Intrinsic resistant



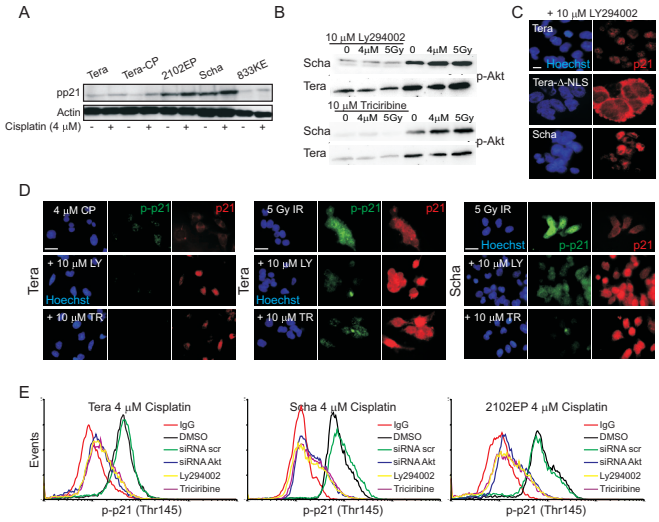
Supplemental Figure 1. (A) IC_{50} values for cisplatin were calculated from the graph in Figure 1A. (B-C) Localization of p21 was determined with immuno-fluorescence; 24h after cisplatin treatment p21 is,cytoplasmic localized in 833KE, using the breast carcinoma cell line MCF-7 as a control for nuclear staining after chemotherapy treatment (59-60) Scale bar, 30 μm. (B). 24h after gamma-irradiation (IR) p21 is localized in the cytoplasm of Tera and Scha (C) Scale bar, 30 μm.



Supplemental Figure 2. (A-C) No significant effect of p21 siRNA on cisplatin induced apoptosis in Tera, Tera-CP and 833KE compared to scr siRNA. Values are the mean ± SD of three independent experiments. Inset shows successful p21 suppression. (D) p21-Δ-NLS is localized in the cytoplasm of Tera-p21-Δ–NLS cells when untreated or treated with cisplatin (CP). Scale bar, 30 μm. (E,F) Tera-p21-Δ-NLS is less sensitive for cisplatin compared to Tera Mock as shown by a lower percentage of apoptotic cells (E) and reduced caspase 3 activation (F) following cisplatin treatment for 24 h. Values are the mean ± SD of three experiments. (G) No significant effect of p21 siRNA on cisplatin induced apoptosis in Tera Mock to scr siRNA. (H) Enhanced caspase 3 activity in Tera-p21-Δ–NLS after treatment with p21 siRNA. Values are the mean ± SD of three experiments.



Supplemental Figure 3. (A) Decreased PARP cleavage after successful downregulation of CDK2 24h after cisplatin treatment of 833KE. (B) Dramatically reduced apoptosis response after co-transfection with CDK2 and p21 siRNA in 2102EP and Scha cells.



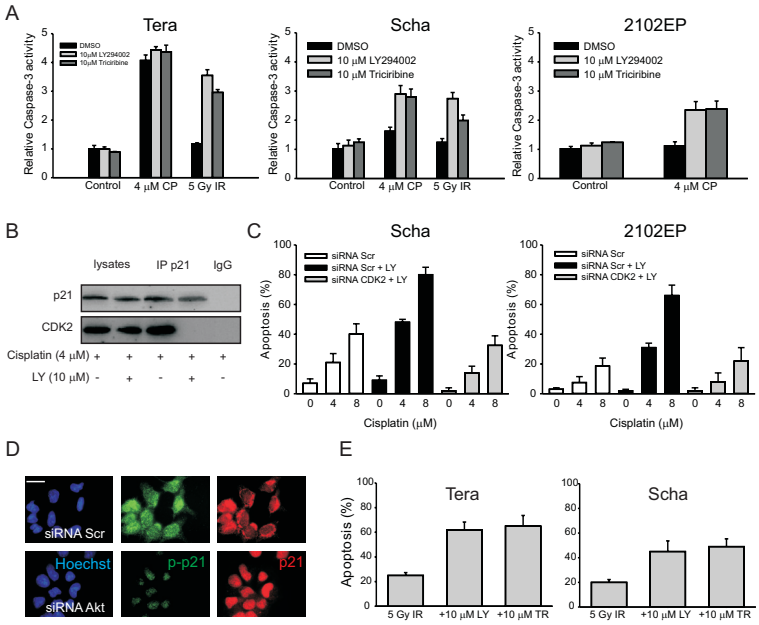
Supplemental Figure 4. (A) Difference in p-p21 levels of the EC cell lines treated and untreated with cisplatin. Note that intrinsic cisplatin-resistant EC cell lines (2102EP and Scha) have higher basal and cisplatin-induced p21/p-p21 levels compared to cisplatin-sensitive EC cell lines. (B) Following treatment with cisplatin or gamma-irradiation of EC cells p-Akt is induced, whereas dephosporylation of p-Akt occurred after treatment with Ly294002 or Triciribine in Scha and Tera. (C) After treatment with 10 μM LY294002 for 24h, p21 is localized in the nucleus of Tera and Scha, whereas this treatment in Tera-p21-Δ–NLS had no effect on p21-Δ-NLS localization. Scale bar, 30 μm. (D) 24h after treatment with gamma-irradiation in combination with either 10 μM LY294002 (LY) or 10 μM Triciribine (TR), phosphorylated levels of p21(Thr145) decreased and p21 is more pronounced localized in the nucleus of Tera and Scha. Scale bar, 30 μm. (E) Dephosporylation of p-Akt and downregulation of Akt reduces the levels of p-p21.

Supplemental Table 1. Patient characteristics

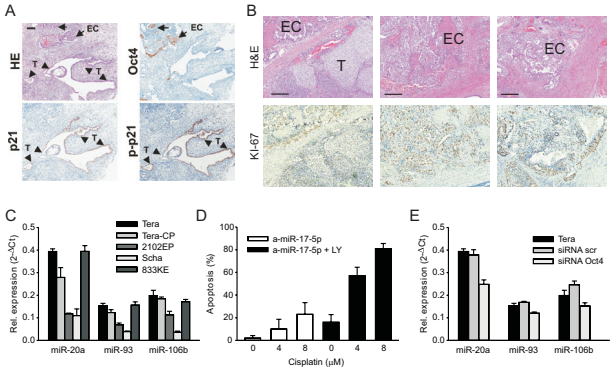
	Chemo-sensitive disease	Refractory disease
No. of patients	31	7
Median age at start of chemotherapy (range)	29 (17-35)	31 (19-44)
Histologie*		
Ec (pure component)	23 (5)	7 (2)
YS (pure component)	6 (1)	1 (0)
ChC (pure component)	6 (0)	2 (0)
T (pure component)	17 (0)	4 (0(
S (pure component)	7 (3)	0 (0)
Prognosis group**		
Good	22	1
Intermediate	5	2
Poor	4	4
Disease outcome		
Death of disease	0	7
Alive with disease	0	0
No evidence of disease	31	0

*single or multiple components; each component listed.

** International Germ Cell Cancer Classification (61).



Supplemental Figure 5. (A) Enhanced caspase 3 activity after the combined treatment of Scha and 2102EP with Ly294002 or Triciribine and cisplatin, while there is no increase in activity in Tera in combination with cisplatin. The combined treatment for 24h of LY294002/Triciribine and gamma-irradiation enhanced caspase 3 activity in both Tera and Scha. (B) Treatment with LY294002 (LY) resulted in the loss of complex formation between p21 and CDK2 in Scha cells. (C) Suppression of CDK2 considerably reduced the increase in apoptosis induction of 2102EP and Scha cells upon combined treatment with cisplatin and LY294002 (LY). (D) Downregulation of Akt reduced the levels of p-p21 and resulted in a more pronounced nuclear localization of p21 in 2102EP. Scale bar, 30 μm. (E) The combined treatment of 24h LY294002/Triciribine and irradiation sensitized both Tera and Scha for gamma-irradiation induced apoptosis. Values are the mean ± SD of three independent experiments.



Supplemental Figure 6. (A) Mature teratoma component (T) stains positive for p21/p-p21 but negative Oct4, whereas the embryonal carcinoma component (EC) stains negative for p21/p-p21 and intensively positive for Oct4 using IHC. A representative sample is shown. Scale bar, 300 μm. (B) Presence of Ki-67 staining indicating proliferation instead of cell cycle arrest in abundant p21 positive EC. (C) Quantitative RT-PCR on miR-106b seed family expression in EC cell lines. Note that the intrinsic cisplatin-resistant EC cell lines (2102EP and Scha) have lower levels of the miR-106b family compared to cisplatin-sensitive EC cell lines (Tera and 833KE). All reactions were run in triplicate. The miRNA expression was normalized to RNU48 expression resulting in a ΔCt from which the $2^{-\Delta Ct}$ value was derived and depicted. (D) Treatment with LY294002 (LY) resensitized synthetic anti-miR-17-5p transfected Tera cells to cisplatin. (E) Quantitative RT-PCR on miR-106b seed family expression in EC cell lines, showing less expression of the miR-106b family in Oct4 suppressed cells, after 48h of treatment with siRNA against Oct4, compared to control.

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Chapter 3

Cisplatin-induced apoptosis and protection against cisplatin are mediated by p53 in a cell context dependent manner in human testicular cancer

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Abstract

In murine testicular carcinoma (TC) cells wild-type p53 contributes to the sensitivity to DNA-damaging drugs in a dose-dependent way. In human TC, however, the role of wild-type p53 functionality in chemotherapeutic response remains elusive. We analysed functionality of wild-type p53 in the unique cisplatin sensitivity of human TC using a short interfering (si)RNA approach. A panel of cisplatin sensitive TC cell lines (833KE and Tera), a subline with acquired cisplatin resistance (Tera-CP) and a panel of intrinsic resistant TC cell lines (Scha and 2102EP), all expressing wild-type p53 were used. Cisplatin sensitivity was related to apoptosis induction in the TC cell lines. P53 and p53-transcriptional targets MDM2 and $p21^{Waf1/Cip1}$ (p21) were expressed in all TC cell lines. Basal levels of MDM2 and p21 mRNA and protein were highest in Scha and 2102EP and depending on p53 transactivation. Following cisplatin exposure, expression levels of p53 increased within 6 h and further enhanced at 24 h with a subsequent increase in MDM2 mRNA and protein levels and Fas cell membrane levels in all TC cell lines. Previously, we demonstrated that high induction of p21 protein levels protected TC cell lines against Fas-mediated apoptosis following irradiation. In contrast, cisplatin treatment resulted in a small induction of p21 mRNA and protein in these TC cells. Down-regulation of p53 with siRNA lowered cisplatin-induced apoptosis in Tera and Tera-CP, which was associated with a diminished Fas membrane expression. In contrast, p53 suppression augmented cisplatin-induced apoptosis in Scha and 2102EP and concomitantly strongly suppressed MDM2 and p21 mRNA and protein expression. Our results indicate that p53 is involved in transactivation of pro- and anti-apoptotic genes in TC cells. The opposite role of p53 in cisplatin-induced apoptosis demonstrates the importance of the cellular context on the p53 transactivation pathway among TC cell lines.

Introduction

Testicular cancer (TC) represents the most frequent solid malignant tumour in men 20-40 years of age (1) and the most frequent cause of death from solid tumours in this age group. The incidence of testicular cancer has been arising world-wide (2). Despite the rising incidence of testicular cancer and the presence of metastatic disease up to 50% at the time of diagnosis (3), a decrease in mortality has been observed thanks to highly effective chemotherapy schemes (2,4). In general, TCs are successfully treated, however, about 20% of patients diagnosed with metastatic disease will not achieve a durable complete remission after initial treatment, either due to incomplete response or relapse, and will eventually die from this disease. Understanding the molecular determinants of chemotherapy sensitivity and resistance in testicular cancer treatment may provide a way to improve chemotherapy sensitivity in other solid tumours.

Two DNA damaging agents, cisplatin and etoposide, are mainly responsible for the successful outcome in TC treatment (5). So far, the analyses of potentially relevant parameters in cisplatin response (detoxification mechanisms, DNA platination and repair, p53 status, Bcl-2 family status) have not elucidated the determinants of sensitivity in human TC cell lines (6-10). Most human TC cell lines, however, show a characteristic hypersensitivity to apoptosis-induction by chemotherapeutic drugs (10-13).

A major role for p53 in the response to chemotherapeutic drugs and the execution of apoptosis has been described (14-18). *P53* is a tumour suppressor gene with a dual role in stress response, regulating a number of genes that co-ordinately force cells into either cell cycle arrest (via trans-activation of $p21^{Waf1/Cip1}$) allowing time for DNA repair or into apoptosis. In turn, the function of p53 is regulated by several mechanisms, acting not only at the transcriptional and translational level, but also influencing the stability of p53 as well as its post-translational modifications and subcellular localisation (19). *P53* is the most frequently mutated gene in human cancers (20), but surprisingly, in human TCs almost no *p53* mutations occur, while the p53 protein is expressed at high levels in the majority of TCs (21). In human ovarian germ cell tumours, displaying high sensitive to cisplatin-containing chemotherapy, no *p53* mutations were detected (22). Despite the still increasing knowledge about the p53 protein as transactivator and cellular gatekeeper for cell growth and division, the effect of wild-type p53 and mutated p53 on drug sensitivity of human TC tumours is still not clear. Several reports have studied chemosensitivity of human TC cell lines in relation to p53 expression, with contradicting results (10,13,23-27).

In order to extensively determine the role of the p53-dependent and p53-independent apoptotic pathway in cisplatin sensitivity and resistance in a setting closely related to the clinic, a well-defined panel of human TC cell lines was used (6,28-30). We recently reported that p53 was upregulated following cisplatin treatment as well as irradiation. However, cisplatin exposure induced apoptosis in TC cells in contrast to irradiation (30). Furthermore, $p21^{Waf1/Cip1}$ (p21) was almost not upregulated in TC cell lines following cisplatin treatment (30). Therefore, we compared p53-dependent cellular and molecular changes with respect to p21 and apoptosis induction by cisplatin in two cisplatin sensitive cell lines (833KE and Tera), a subline of Tera with acquired resistance to cisplatin (Tera-CP) and two intrinsic cisplatin resistant cell lines (Scha and 2102EP).

Materials and methods

DNA and Chemicals. RPMI 1640 medium was obtained from Gibco (Paisley, Scotland) and foetal calf serum from Sanbio (Uden, the Netherlands). Cisplatin was purchased from Bristol-Myers Co. (Weesp, the Netherlands). 3-(4,5-dimethylthiazol-2-yl) 2,5-diphenyltetrazolium bromide (MTT) was obtained from Sigma (St.Louis, Mo).

Cell Lines. For the cell lines used in this study, origin and pre-treatment were described by Sark et al (28). 2102EP was a gift from L. Looijenga, (Erasmus University, Rotterdam, the Netherlands). The testicular cancer cell lines Tera, Tera-CP, 833KE, 2102 EP and Scha and the human ovarian carcinoma cell line A2780, used as a control for different protein, were cultured in RPMI 1640 medium supplemented with 10% foetal calf serum at 37oC in a humidified atmosphere with 5% CO2. All cell lines grew attached, 833KE was harvested by treatment with protease XXIV 0.005% for 3 min, 2102EP was harvested by treatment with trypsin and Tera, Tera-CP and Scha were harvested by scraping.

RNA interference. p53 I small interfering RNA (siRNA) molecules, sequences 5'-GCA UGA ACC GGA GGC CCA UdTdT-3' (sense) and 5'-AUG GGC CUC CGG UUC AUG CdTdT-3' (anti-sense), p53 II siRNA, sequences 5'-CUU CGA CUU UGU CAC CGA GdTdT-3' (sense) and 5'-CUU ACG CUG AGU ACU UCG AdTdT-3' (anti-sense) and negative control (scrambled) were purchased from Eurogentec (Maastricht, the Netherlands). TC cells (0.4 x 106/ well) were transfected in 6 well plates with 5 µl of 20 µM siRNA duplexes using Oligofectamine reagent according to the manufacturer's instructions (Invitrogen BV, Breda, the Netherlands). After 24 h, cells were treated with cisplatin. At 6 h or 24 h after the treatment cells were harvested for protein isolation. Alternatively, in order to perform an apoptosis assay, at 24 h after transfection, cell were harvested and plated in 96-well plates. The day after, cells were treated with cisplatin. At 24 h after the treatment, the percentage of apoptotic cells was determined by acridine orange apoptosis assay.

Drug Sensitivity Assay. Drug sensitivity testing was performed with the microculture tetrazolium assay as described previously (28). The linear relationship of cell number to MTT formazan crystal formation and the exponential growth of cells in the wells were checked. Cytotoxicity for all cell lines was determined after 4 days. Each drug concentration was tested in quadruplicate.

Western Blotting. After 6 hrs or 24 h cisplatin incubation cell were harvested and lysates were examined by Western Blot analysis as described previously (30). Immunodetection of p53, p21, MDM2, poly-(ADP-ribose)-polymerase (PARP) was performed with the following antibodies:anti-p53-DO-1 (clone sc-126; Santa Cruz Biotechnology, CA, USA), anti-phospho-p53 (ser15 and ser46; Cell Signaling, MA, USA) mouse anti-MDM2 (SMP14, Oncogene Research Products; San Diego, USA), mouse anti-p21 (EA10, Oncogene Research Products), rabbit anti-Parp (Roche Applied Science, Almere, the Netherlands) and mouse anti-ß-actin (C4, MP Biomedical, Eindhoven, the Netherlands). The antibody binding was eventually determined using horseradish peroxidase (HRP)-conjugated secondary antibodies (all from DAKO, Glostrup, Denmark) Chemiluminescence was detected with the BM Chemiluminescence detection kit (Roche Applied Science). Membranes were exposed to Kodak X-OMAT films. Equal protein loading was checked for with Ponceau S and ß-actin staining.

Apoptosis. For apoptosis measurements cells were plated in 24-well culture plates. Cells were continuously incubated with cisplatin at various concentrations. Acridine orange fluorescent staining of nuclei in unfixed cells was used to distinguish apoptotic from vital cells (6). Staining was performed 6, 24, 48 and 72 h after start of incubation. Results are expressed as the percentage of apoptotic cells in a culture by counting at least 300 cells per well.

Caspase 3 activity. After 6 hrs or 24 h cisplatin incubation, cells were harvested as usual and centrifuged at 1400 rpm for 8 min. After one wash step with cold PBS 1x, cells were resuspended in 50 µl of chilled Cell Lysis Buffer [10 mM HEPES, 2 mM EDTA, 0.1% CHAPS / NP40, 5 mM DTT, 1 mM PMSF (10 µg/ml pepstatin A) (20 µg/ml leupeptin) (10 µg/ml aprotinin)] and incubated on ice for 10 min. Subsequently cell lysates were centrifuged in a microcentrifuge at 10,000 g for 3 min at 4□C to precipitate cellular debris. The supernatants were then transferred to new microcentrifuge tubes. From each sample, 5 µl were used to perform Bradford protein quantification. Samples were then diluted to the final protein concentration of 1 µg/µl for caspase assay, with lysis buffer. 25 µl

of 2X Reaction Buffer [31.25% sucrose, 0.3125% CHAPS (3-[3-cholamido-propyl)-dimethammonio]-1propane-sulphonate), HEPES: 312.5 mM (pH 7.5 Mw: 238.31g/l)] (containing DTT and caspase 3/7 substrate DEVD-AFC) were added to each 25 µl sample in a 96-wells plate (Cliniplate flat bottom black) and incubated for 1 h at 37□C. Fluorescent detection of protease activity was performed by means of a fluorometer equipped with a 400-nm excitation filter and 505-nm emission filter. Fold-increase in protease activity was determined by comparing these results with the level of the control.

Detection of Fas membrane expression. Cisplatin-treated or untreated cells were stained with a phycoerythrin (PE)-conjugated Ab against Fas (DX2 from Becton Dickinson, Erembodegem-Aalst, Belgium) for 1 hour at room temperature. Subsequently, cells were washed and analysed by flow cytometry (FACS-Calibur; Becton Dickinson). The mean fluorescence intensity was determined by comparison of the fluorescence intensity with the fluorescence intensity of isotype control cells.

Quantitative real-time PCR for p21 and MDM2. Total RNA was isolated using the RNeasy Midi Kit according to the manufacturer's instructions (Qiagen, Venlo, the Netherlands). cDNA was synthesized from total RNA as described by the manufacturer's protocol (Life Technologies, Invitrogen) using oligo dT primers and M-MLV transcriptase. Quantitative real-time PCR was performed using SYBR Green qPCR SuperMix (Invitrogen). p21 and MDM2 mRNA levels were normalized to the level of GAPDH in the same sample. Results of at least 3 experiments in duplicate are expressed as mean ± SD. Used primers: GAPDH For: CAC CAC CAC GGA GAA CGC TGG, GAPDH Rev: CCA AAG TTG TCA TGG ATG ACC, P21 For: CCT GTC ACT GTC TTG TAC CCT, P21 Rev: GCG TTT GGA GTG GTA GAA ATCT, MDM2 For: TCC CAA TTA GCT TGG CCT AC, MDM2 Rev: TAA CAC GGT GAA ACC CTG TC.

Results

Expression of p53 and the p53 transactivated proteins MDM2 and p21. All TC cell lines express wild-type p53 (31). Basal p53 levels are highest in 833KE, followed by Scha, Tera and Tera-CP. Expression levels of MDM2, an important target of p53 transactivation, were higher in 833KE and Scha compared to Tera and Tera-CP. P21, another target of p53 transcriptional activation was expressed in Scha, and minimally detectable in 833KE, Tera and Tera-CP (Figure 1). The human A2780 ovarian cancer cell line was used as a positive control for wild-type p53 expressing cells (30). These results suggest there is a relation between constitutively high wild-type p53 and MDM2 expression levels in 833KE and Scha compared to Tera or Tera-CP, however this relation was not observed for p21.

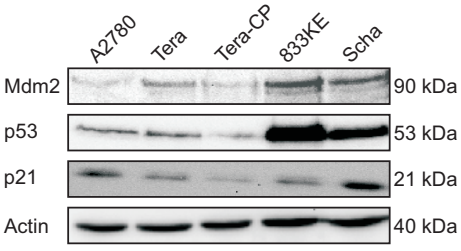

Figure 1. Large differences in p53, p21 and MDM2 expression levels between TC cell lines. Expression levels in TC cells were determined using western blot of proteins in whole-cell lysates. Human A2780 ovarian cancer cells were used as control for wild-type p53 expressing cells. Immunoblotting was performed as described in "Material and Methods". Each lane was loaded with 20 µg protein. Actin was used as a loading control. A representative example of at least three independent experiments is shown.

Effect of cisplatin treatment on the expression of p53, p21and MDM2 in TC cells. To determine whether cisplatin treatment induces expression of p53, cells were incubated for 6 h and 24 h with cisplatin concentrations ranging from the ID_{50} of highly sensitive cell lines to more than the ID_{90} of all cell lines used in the present study (Table 1). In addition, we

measured the drug-induced protein expression of genes, which are transcriptionally activated by wild-type p53, i.e. p21 and MDM2. Within 6 h a concentration-dependent increase in p53 expression levels was observed in the TC cell lines (results not shown), however, no changes in expression of p21 and MDM2 were observed (results not shown). A prolonged incubation with cisplatin for 24 h resulted in a concentration-dependent increase in cellular p53 levels in the TC cell lines (Figure 2). The p53 induction levels in the TC cell lines after treatment with cisplatin for 24 h were higher than p53 levels after a 6 h treatment (results not shown). With lower cisplatin concentration for 24 h, the increase in p53 was more pronounced in 833KE than in Scha cells, while at higher cisplatin concentrations a similar induction of p53 was found. As to the isogenic cisplatin resistant Tera/Tera-CP model, a stronger induction of p53 was detectable in Tera and Tera-CP after cisplatin treatment for 24 h, with a more pronounced induction in Tera at lower cisplatin concentrations. Note that the constitutive level of p53 was at least 5-fold higher in 833KE and Scha, indicating that the absolute cellular p53 expression levels after cisplatin induction are comparable between the TC cell lines.

MDM2 levels increased in all TC cell lines after a 24 h treatment with increasing cisplatin concentrations (Figure 2). At the highest cisplatin concentration used, MDM2 levels slightly declined in the TC cell lines, except for Scha. Despite the high induction of p53 and MDM2, a minor induction or even no induction of p21 was detectable in the TC cell lines (Figure 2). These results are in agreement with our previous study showing that irradiation but not cisplatin strongly induces p21 mRNA and protein expression demonstrating that p21 can be induced depending on the type of DNA damage in TC cell lines (30-31).

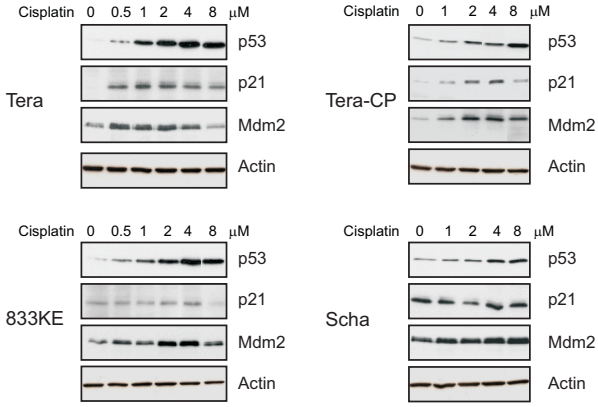

Figure 2. Induction of p53, p21 and MDM2 protein levels in TC cells after cisplatin treatment for 24 h. Cells were treated with increasing concentrations of cisplatin for 24 h. Immunoblotting was performed as described in "Material and Methods". Each lane was loaded with 20 μg protein. Actin was used as a loading control. A representative example of three independent experiments is shown. To detect p21 in lysates of Tera, Tera-CP, and 833KE membranes had to be exposed to X-ray films longer compared to Scha.

Cisplatin induced apoptosis in TC cells. Despite the induction of p53, no PARP cleavage nor apoptotic cells, defined as cells with condensed and fragmented nuclei, were observed after 6 h (data not shown). However, treatment of cisplatin for 24 h resulted in a massive induction of apoptosis. Apoptosis was also reflected in caspase 3 activation, and cleavage of PARP in all TC cell lines in a cisplatin concentration-dependent manner (Figure 3A-C). For Scha and Tera-CP higher cisplatin concentration were needed compared to 833KE and Tera. The results of the different assays were in accordance with each other showing that Tera and 833KE are the most sensitive cell lines, while Tera-CP and Scha were relatively resistant to cisplatin (Figure 3A-C).

Influence of p53 down-regulation on response to cisplatin treatment. The minimal induction of p21 suggested that p53 is not functional in TC cells following cisplatin treatment.

To study the role of p53 in more detail, we suppressed p53 expression with p53 siRNA. When p53 suppressed TC cells were treated with cisplatin, different effects on apoptosis were observed (Figure 4). Down-regulation of p53 makes the cisplatin-sensitive Tera partially resistant to cisplatin-induced apoptosis compared to both untransfected and scr siRNA transfected Tera. Similar observations were made for Tera-CP, except that these cells had to be treated with cisplatin for 48 h and the effect of p53 siRNA was slightly less pronounced in Tera-CP. No effect of p53 suppression was observed in 833KE (results not shown). In contrast, the down-regulation of p53 sensitized Scha to cisplatin-induced apoptosis compared to control cells and scr siRNA transfected cells. This sensitizing effect was detectable after 3 h and 6 h of cisplatin treatment (data not shown) and more pronounced after 24 h (Figure 4A). We added 2102EP, an additional intrinsic cisplatin-resistant human TC cell line known to be wild-type p53 and to express relatively high levels of p21 (31), to our panel to extent our findings of the protective role of p53 in cisplatin-resistant TC cells.. Following downregulation of p53, a similar sensitization to cisplatin was observed in 2102EP as in Scha (Figure 4).

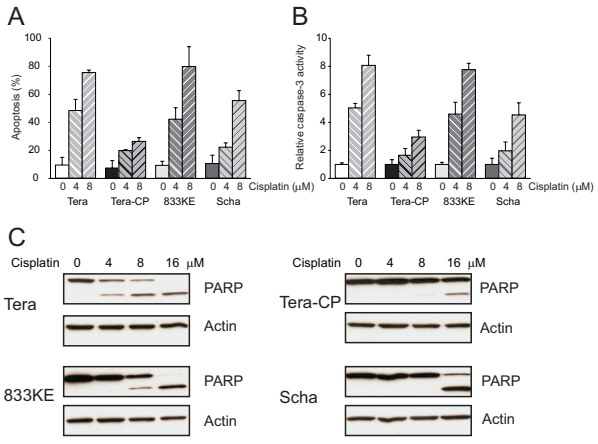

Figure 3. Cisplatin treatment for 24 h resulted in apoptosis in TC cells. Apoptosis was determined with several different assays using cell morphology, caspase 3 activity and PARP cleavage as read-outs. Cisplatin concentration-dependent apoptosis (0, 4 and 8 μM) is related to the cell survival (see table 1). (A) The percentage of apoptotic cells was determined with fluorescence microscopy on acridine orange stained cells, (B) caspase 3 activity was determined in whole cell lysates using a fluorescence assay, and (C) PARP cleavage was determined with immunoblotting on whole-cell lysates after exposure of the cells to cisplatin. Counting of apoptotic cells, caspase 3 activity assay and immunoblotting were performed as described in "Materials and Methods". Values are the mean ± SD of three experiments. *$p < 0.05$; **$p < 0.01$; ***$p < 0.005$; compared to matching siRNA scrambled (scr) transfected cells.

At the protein level, down-regulation of p53, MDM2 and p21 was observed in p53 siRNA treated cells both in the absence of cisplatin and after addition of cisplatin for 24 h compared to control cells and scr siRNA transfected cells. Although treatment of p53 siRNA transfected cells with a high cisplatin dose induced p53 as well as MDM2 and p21 protein expression, these levels still remains lower when compared to the corresponding levels in untransfected or scr siRNA transfected cells treated with cisplatin (Figure 4B).

To strengthen the p53 dependent effect of the siRNA approach, we used two different p53 siRNAs. The caspase activity assay results were in agreement with those obtained with the apoptosis assay. A clear induction of caspase 3 activity was observed in all four cell lines treated with 4 μM cisplatin. Differences in cisplatin-induced caspase 3 activity reflect the differences in cisplatin concentration dependent apoptosis-induction between the four cell lines. A 2 to 2.5-fold lower caspase activity was observed in p53 suppressed Tera and Tera-CP cells

after cisplatin treatment compared to scr siRNA transfected cells (Figure 5A). In contrast, in cisplatin-treated p53 suppressed Scha and 2101EP cells a 2 to 2.5-fold higher caspase activity was observed compared to scr siRNA transfected cells (Figure 5A). In Tera-CP, p53 siRNA strongly reduced the p53 expression level and less PARP cleavage was observed following cisplatin treatment. In 2102EP, a similar reduction in p53 was observed but now more PARP cleavage was observed following cisplatin treatment (Figure 5B). Similar effects were observed with either of the two p53 siRNAs in the caspase 3 activity assays and with western blotting (Figure 5A and B).

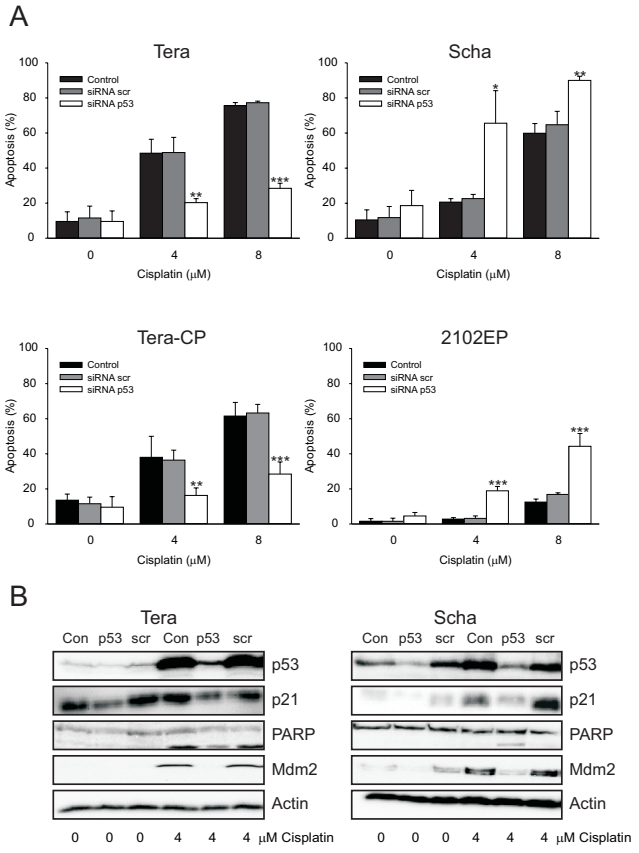

Figure 4. Suppression of p53 has opposite effects on cisplatin-induced apoptosis in different TC cell lines. TC cells were either untransfected (con), or transfected with p53 siRNA (p53) or scr siRNA (scr) and after 24 h treated with cisplatin (0, 4, or 8 μM) for an additional 24 h. (A) Apoptotic cells were visualised with fluorescence microscopy on acridine orange stained cells with acridine orange using a fluorescence microscopy counted with on stained cells. Values are the mean ± SD of three experiments. *p < 0.05; **p < 0.01; ***p < 0.005; compared to matching siRNA scrambled (scr) transfected cells. (B) Transfection with p53 siRNA suppressed p53, p21 and MDM2 expression in Tera and Scha cells also after cisplatin treatment (4 μM) for 24 h. PARP cleavage, however, was reduced in p53 suppressed Tera cells but enhanced in p53 suppressed Scha cells following cisplatin treatment. Protein expression was determined in whole-cell lysates using immunoblotting as described in "Material and Methods". Each lane was loaded with 20 μg protein. Actin was used as a loading control. To detect p21 in lysates of Tera membranes had to be exposed to X-ray films longer compared to Scha. A representative example of three independent experiments is shown.

Influence of p53 down-regulation on p21 and MDM2 mRNA and Fas membrane expression. Since p53 acts as a transcription factor of p21 and MDM2, we analysed the effect of p53 suppression on mRNA levels of both proteins using quantitative PCR. Downregulation of p53 had almost no effect on basal p21 and MDM2 mRNA levels in Tera and Tera-CP, whereas an apparent effect on p21 and MDM2 mRNA levels was found in Scha and 2102EP. Treatment with cisplatin induced mRNA expression of both mRNAs in all TC cell lines,

which was almost completely blocked in p53 suppressed cells (Figure 6A and B). Previously, we have shown that cisplatin-induced apoptosis is depending on activation of the Fas/FasL system in the cisplatin-sensitive Tera and 833KE cells, while activation of the Fas/FasL system is inhibited in the cisplatin-resistant Tera-CP and Scha cells (29). Fas is induced in a p53-dependent manner and, in addition, p53 is involved in the translocation of Fas to the cell surface (29,30,32-36). Therefore, we monitored the effect of p53 siRNA on Fas membrane expression. In agreement with our previous results, cisplatin strongly induced Fas membrane expression in Tera and to a lesser extent in Tera-CP, Scha and 2102EP. The upregulation was p53-dependent, since downregulation of p53 with p53 siRNA resulted in a strong inhibition of cisplatin-induced Fas membrane expression in all cell lines (Figure 6C). Notably, the effect of p53 suppression on Fas membrane expression in the absence of cisplatin was less evident. These results indicate that p53 is involved in basal transcription and cisplatin-induced transcriptional activation of p21and MDM2 and Fas membrane expression in a cell context dependent manner.

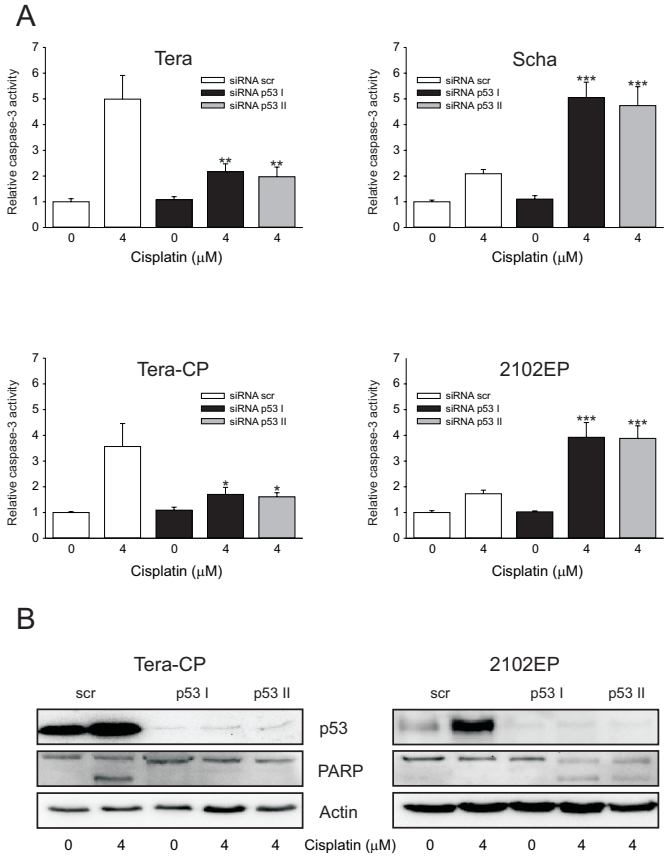

Figure 5. Suppression of p53 has opposite effects on cisplatin-induced caspase 3 activity in different TC cell lines. Cells were either transfected scr siRNA (scr), and with p53 siRNA set I (p53 I) or p53 siRNA set II (p53 II). Following siRNA treatment for 24 h, cells were treated with cisplatin (4 μM) for an additional 24 h. (A) Caspase 3 activity was determined in whole cell lysates using a fluorescence assay as described in "Material and Methods". Values are the mean ± SD of three experiments. Values are the mean ± SD of three experiments. *p < 0.05; **p < 0.01; ***p < 0.005; compared to matching siRNA scrambled (scr) transfected cells. (B) Both p53 siRNA sets suppressed p53 expression in Tera-CP and 2102EP cells, also after cisplatin treatment (4 μM) of the p53 suppressed cells for 24 h. PARP cleavage, however, was reduced in p53 suppressed Tera-CP cells and enhanced in p53 suppressed 2102EP cells following cisplatin treatment. Protein expression was determined in whole-cell lysates using immunoblotting as described in "Material and Methods". Each lane was loaded with 20 μg protein. Actin was used as a loading control. A representative example of three independent experiments is shown.

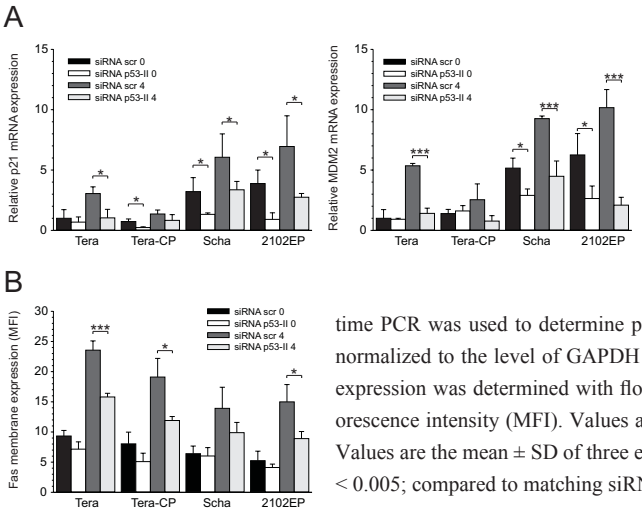

Figure 6. Cisplatin-induced p21 and MDM2 mRNA and Fas surface expression in TC cells is strongly reduced in p53 suppressed TC cells. Cells were either transfected with p53 siRNA (p53 I) or scr siRNA (scr). Following siRNA treatment for 24 h, cells were treated with cisplatin (4 μM) for an additional 24 h. (A) Quantitative real-time PCR was used to determine p21 and MDM2 mRNA levels that were normalized to the level of GAPDH in the same sample. (B) Fas membrane expression was determined with flow cytometry and indicated as mean fluorescence intensity (MFI). Values are the mean ± SD of three experiments. Values are the mean ± SD of three experiments. *$p < 0.05$; **$p < 0.01$; ***$p < 0.005$; compared to matching siRNA scrambled (scr) transfected cells.

Discussion

In the present study, we demonstrate that p53 is functional in most TC cell lines, but its involvement in the response to cisplatin, i.e. pro-apoptotic or anti-apoptotic involvement, is cell context dependent. It is shown here that in a panel of cisplatin-sensitive and -resistant human TC cell lines cisplatin-induced apoptosis induction is related to the difference in cisplatin sensitivity between TC cell lines. Functionality of p53 was demonstrated by p53 siRNA, which suppressed basal p53 as well as p21 and MDM2 protein and mRNA expression levels in TC cell lines. Our results further showed that cisplatin treatment resulted in enhanced wild-type p53, p21 and MDM2 mRNA and protein expression and Fas cell surface expression, which was prevented by p53 suppression in most TC cell lines. Remarkably, inhibition of cisplatin-induced p53 expression with p53 siRNA causes a decrease in apoptosis levels in the cisplatin-sensitive Tera cell line and its cisplatin-resistant subline Tera-CP and an enhanced induction in apoptosis in two intrinsic cisplatin-resistant cell lines, 2012EP and Scha, but had no effect on cisplatin sensitivity of 833KE.

In this study, we have demonstrated that higher basal levels of wild-type p53 in TC cells were accompanied by higher MDM2 and p21 protein and mRNA levels. Moreover, we showed that down-regulation of p53 using an siRNA approach resulted in a lower expression of MDM2 and p21 mRNA, suggesting the presence of a transcriptionally active p53 in human TC cell lines. Similar observations were made in another testicular cancer study, describing a correlation between p53 protein level in vivo and MDM2 mRNA expression (37). Next, we detected a concentration-dependent induction of p53 and MDM2 in TC cells following cisplatin treatment. The cisplatin concentration-dependent induction of p21 in the TC cell lines was much less clear. This finding is in agreement with a recent report in which we described that γ-radiation but not cisplatin massively induced p21 mRNA and protein expression in TC cells (30). Other studies have shown that in addition to γ-radiation (30,38), etoposide treatment (23) also resulted in an induction of p21 protein in human TC cell lines. These observations can be explained by a different mechanism of p53 activation after DNA damage induced by either γ-radiation or etoposide versus cisplatin.

Several post-translational modification of the p53 protein may play a role in determining its functional activity. Phosphorylation of p53 at ser15 blocks p53 binding to MDM2

leading to the accumulation and activation of p53, whereas phosphorylation of p53-ser46 positively regulates apoptosis-induction in response to DNA damage (39-43). We only determined basal levels of p53 phosphorylation but did not find any difference between the cell lines (results not shown). Furthermore, conflicting data about the importance of these modifications have been published (44,45). Other p53 regulatory proteins have been suggested to be more important such as the related proteins MDM2 and MDM4 (MdmX) (45-47). The role of p53-MDM2 interaction in the response of testicular cancer to cisplatin is still not conclusive (48,49). However, treatment of the cells with nutlin-3, a small molecule antagonist of MDM2, was sufficient to induce apoptosis in TC cells suggesting that p53 activity is negatively regulated by MDM2. In addition, it was shown that MDM4 also functions as a negative feedback regulator of p53 in some of the TC cell lines (50). When nutlin-3 was combined with cisplatin an enhanced reduction in cell survival was observed in TC cells. Unfortunately, no causal relation between the effect of nutlin-3 or cisplatin with the presence of functional active p53 was established (50).

Table 1. The concentration of cisplatin inhibiting cell survival by 50 % after continuous treatment with cisplatin in the MTT assay (ID_{50} cisplatin) and the protein expression characteristics of TC cell lines.

	ID_{50} Cisplatin (μM)[1]	p53	MDM2 (protein expression)[2]	p21	Fas (membrane)[3]
Tera	0.7 ± 0.1	+	+	+	+
Tera-CP	2.1 ±0.2	+	+	+	+
833KE	1.0 ± 0.2	++	++	+	+
Scha	3.3 ± 1.0	++	++	++	+
2102EP	4.1 ± 0,7	++	++	++	+

1) ID_{50} values are expressed as means ±SD of three independent experiments performed in quadruplicate using the MTT assay.

2) Protein expression as detected with western blotting and from refs (31,50)

3) Fas membrane expression levels as detected with flow cytometry

Although clinical analyses of testicular cancers revealed almost no TP53 mutations (20), another report showed that mutant p53 was present in a subset of chemoresistant testicular cancers (24), further supporting the hypothesis of a functional p53 in human testicular cancer. A relation between the presence of wild-type p53, the susceptibility for apoptosis-induction and the chemosensitivity of a panel of TC cell lines has been observed in several studies (23,29,51), but these results were challenged by other studies using either a different TC cell line panel (25) or p53 inhibition by human papilloma virus type-16 E6 in TC cells (13). Therefore, we have used an siRNA approach to investigate the role of p53 in cisplatin-induced expression of MDM2, p21 and Fas and in cisplatin-induced apoptosis. Although p53 suppression reduced expression of these genes in all TC cell lines, an opposite effect on cisplatin-induced apoptosis was observed. Down-regulation of p53 led to a decrease in cisplatin sensitivity in Tera cells. In the cisplatin-resistant subline Tera-CP, a similar effect of p53 was observed but only after prolonged incubation with cisplatin, in line with the mechanism of acquired cisplatin-resistance in these cells (6). Our data are in agreement with a recent micro-array study from Kerley-Hamilton et al (52) in which the authors demonstrated

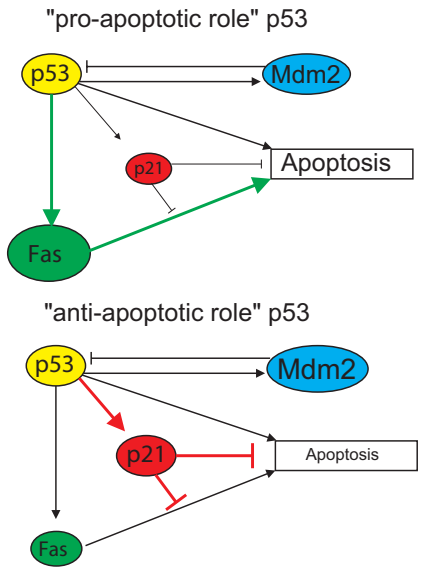

Figure 7. Proposed simplified model showing the mechanisms through which p53 can either be involved in a pro- or anti-apoptotic response to cisplatin treatment in TC cells. Cisplatin-induced DNA damage activates p53, which in turn transcribes p21 and MDM2, and induces Fas membrane expression. Recently, we have identified the Fas pathway to be involved in cisplatin-induced apoptosis in cisplatin-sensitive TC cell lines (29). Moreover, we demonstrated that high cytoplasmic p21 expression inhibits both Fas death receptor mediated apoptosis (30) and cisplatin-induced apoptosis (31). Here, we propose the following model. In TC cells hardly expressing p21, cisplatin-induced p53 activation results in enhanced Fas death receptor expression and Fas death receptor mediated apoptosis. In addition, cisplatin-induced activation of p53 may also lead to enhanced intrinsic (mitochondrial) apoptosis. Thus, p53 has a pro-apoptotic function. In TC cells expressing relatively high cytoplasmic p21 levels, cisplatin-induced p53 activation also results in enhanced Fas death receptor expression, although to a lesser extent. However, the relatively high cytoplasmic p21 expression levels are strongly depending on p53 transcriptional activity and are blocking both cisplatin-induced extrinsic and intrinsic apoptosis. In these cisplatin-resistant TC cells, p53 has an anti-apoptotic function as transcriptional activator of p21 and possibly MDM2.

that p53 was directly involved in cisplatin sensitivity in Tera cells, as the down-regulation of p53 corresponded to the global decrease of several cisplatin-induced genes involved in apoptosis and in particular of genes involved in the extrinsic apoptosis machinery, like *FAS*, Fas adaptor *LRDD* and a gene involved in positive Fas regulation, *PHLDA3* (52). Previously, we have demonstrated that the Fas/FasL system is active and functional in the cisplatin-sensitive TC cell lines, including Tera cells (29). An additional factor sensitizing these TC cells to cisplatin and Fas mediated apoptosis is the low expression level of cytoplasmic p21 (30,31). Down-regulation of p53 prevented cisplatin-induced Fas surface expression in these cells. We therefore, hypothesize that in TC cells with low p21 expression levels, p53 is a pro-apoptotic determinant of cisplatin-induced apoptosis via the Fas death receptor but p53-mediated effects via the intrinsic pathway can not be excluded (Figure 7). In intrinsic cisplatin-resistant TC cell lines, expressing high p21 and relatively low levels of Fas, we observed an anti-apoptotic role for p53. Suppression of p53 resulted in reduced expression levels of both p21 and Fas. Recently, we have demonstrated that cytoplasmic localized p21 is a key determinant of cisplatin-resistance in TC cell lines and in cisplatin refractory testicular cancer patients. An important regulatory mechanism of p21 protein expression was identified and involved binding of miR-106b seed family members to the 3'-UTR of p21, and thus reduced expression of p21. Expression levels of miR-106b family members were much higher in cisplatin sensitive compared to intrinsic cisplatin-resistant TC cell lines explaining the high p21 protein levels in the latter (31). Our present results indicate that in intrinsic cisplatin-resistant TC cells p53 plays an anti-apoptotic role due to its effect as transcriptional activator on p21 mRNA and thus on p21 protein levels (Figure 7).

In conclusion, our results suggest a dual role for p53 in transactivation and cisplatin-induced apoptosis in TC cell lines depending on the cellular context. The observed effect of p53 on cisplatin-induced apoptosis may be closely related to p21 and Fas expression levels in TC cells.

Acknowledgment
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Chapter 4

Disruption of the MDM2-p53 interaction strongly potentiates p53-dependent apoptosis in cisplatin resistant human testicular carcinoma cells via the Fas/FasL pathway

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Jourik A. Gietema and Steven de Jong

Manuscript submitted

Abstract

A major role for p53 in the response and execution of apoptosis after chemotherapy has been described for many cancers. Although testicular cancer (TC) often express high levels of p53 and almost no TP53 mutations are found, resistance to chemotherapy is still observed. In this study, we demonstrate that in intrinsic and acquired-cisplatin resistant TC cells p53 resides in a complex with murine double minute 2 (MDM2) after cisplatin treatment. Hyper-activation of the p53 pathway using the MDM2 antagonist Nutlin-3 in TC cells leads to nuclear localization of p53 and effective apoptosis induction as single agent. Targeting MDM2 with Nutlin-3 or short interfering RNA extremely sensitizes resistant TC cells to cisplatin. The observed effects are dependent on the presence of wild-type p53, since mutant p53 expressing TC cells or wild-type p53 suppressed TC cells are resistant to Nutlin-3. These results indicate that targeting the p53/MDM2 axis, in combination with standard treatment, appears to be a powerful strategy to pursue in cisplatin-resistant or -refractory testicular cancers. Specifically, we show that the Fas death receptor pathway plays an important role in MDM2 antagonist-induced apoptosis in TC cells. Importantly, we have identified a similar mechanism in Hodgkin lymphoma and acute myeloid leukaemia cells, suggesting a common profile for Nutlin-3-induced apoptosis, which involves the Fas death receptor pathway.

Introduction

Most testicular cancer (TC) patients respond well to cisplatin-based chemotherapy, however, there is still a subset of these young patients that will die because of chemo-resistant or chemo-refractory disease (1-3). Similar to its effects in patients, cisplatin proved to be an extremely cytotoxic drug, inducing massive apoptosis in TC cell lines (4-8). An important role for p53 in the response to chemotherapeutic drugs and the execution of apoptosis has been described (9-13). p53 is a tumour suppressor protein with a dual role in stress response by transactivation of genes that induce apoptosis, such as *FAS*, as well as genes that induce cell-cycle arrest, such as *CDKN1A* (encoding $p21^{cip1/wafl}$), allowing time for DNA repair. The function of p53 is regulated by several mechanisms, acting not only at the transcriptional and translational level, but also on stability, post-translational modification, and subcellular localization of p53 (14,15).

P53 is the most frequently mutated gene in human cancers (16-19) but surprisingly, in human TCs almost no *p53* mutations occur and the p53 protein is expressed at high levels in the majority of TCs (20-22). Despite the increasing knowledge about p53 as a transactivator and cellular gatekeeper for cell growth and division, the effects of wild-type p53 (and mutated p53) on drug sensitivity of human tumours is still not clear. Recently, we showed in TC cell lines, that p53 function is related to the response to cisplatin-induced DNA-damage, which also involved activation of the Fas death receptor pathway (4). Several studies have reported the effect of wild-type p53 expression on chemo-sensitivity of human TC cell lines with contrasting and sometimes conflicting results (6,23-31).

Tumours that retain wild-type p53 are supposed to have other defects in the p53 pathway, such as presence of miR-371-373, miR-106b seed family, cytoplasmic p21, lack of PTEN expression or increased MDM2 expression (32-36). MDM2, as transcriptional target of p53, is the main negative feedback regulator of p53. By binding to the transactivation domain of p53, MDM2 is able to regulate p53 activity and stability via several mechanisms such as promoting p53 degradation through ubiquitination, stimulating p53 nuclear export, and inhibiting acetylation of p53 (15,37,38).

Inhibition of the p53-MDM2 interaction, with small molecules like RITA and Nutlin-3, provides an attractive strategy for (re)activating wild-type p53 in a non-genotoxic way. This (re)activation leads to cell cycle arrest and or apoptosis in tumour cells with wild-type p53 (39-43). Restoration of p53 function by Nutlin-3 may thus have profound therapeutic use for tumours that have retained wild-type p53, particularly if MDM2 activity is disproportionally increased. Recently, Nutlin-3-induced apoptosis was investigated in a small panel of TC cell lines, and only additive effects were seen in combination with cisplatin. However, no mechanistic insights in Nutlin-3-induced apoptosis were offered (43,44). To further explore the potential of MDM2 inhibition as a mean to activate p53 in TC and to determine underlying mechanisms, we have determined the role of p53 and MDM2 in cisplatin-induced apoptosis using cisplatin-sensitive and -resistant human TC models. Finally, the importance of the Fas death receptor pathway in Nutlin-3 induced apoptosis has been studied.

Materials and Methods

Cell Lines & reagents. A well defined panel of cisplatin sensitive and resistant EC cell lines 833KE, Tera, Tera-CP, Scha and 2102EP (all expressing wild-type p53) and NCCIT (mutant p53) were used in this study (4,21,24,45,46). Tera, Tera-CP, 2102EP, Scha, 833KE and NCCIT were cultured in RPMI 1640 medium (Gibco, Invitrogen, Merelbeke, Belgium) supplemented with 10% fetal calf serum (Bodinco, Alkmaar, the Netherlands) at 37oC in a humidified atmosphere with 5% CO2. Tera, Tera-CP and Scha were harvested by scraping, 2102EP and NCCIT by treatment with trypsin and 833KE by treatment with 0.005% protease XXIV (Sigma, Amsterdam, the Netherlands). The cell lines OCI-AML-3, MOLM-13, HL-60, KM-H2, L540 and L428 were maintained as described previously (41,42,47). Cisplatin was purchased from Bristol-Myers Co. (Weesp, the Netherlands), Nutlin-3 from Cayman Chemical (Huissen, the Netherlands). To block CD95/CD95L interactions, cells were incubated with anti-CD95L Ab Nok-1 (Becton Dickinson, Breda, the Netherlands) and azide free IgG control (Becton Dickinson).

Drug Sensitivity Assay. Drug sensitivity testing was performed with the microculture tetrazolium assay as described previously (4).

Immunofluorescence. Cells were seeded on 0.01% poly-L-lycine (Sigma) precoated coverslips. 24h after indicated treatment cells were fixed with Methanol/Acetone (1:1) for 30 min at RT and then blocked with 1% bovine serum albumin and 1% normal goat serum in PBS for 30 min at RT. Followed by immunostaining with the corresponding antibodies and counterstained with Alexa-Fluor goat secondary antibodies (Molecular Probes, Invitrogen). Finally, cells were stained with Hoechst 33258 (Molecular Probes, Invitrogen) for 5 minutes, washed with PBS, and coverslips were mounted on slides with Vectashield (Vector Laboratories, Amsterdam, the Netherlands). After staining cells were analyzed using a Quantimet 600S digital analysis system (Leica Microsystems).

Immunoprecipitation. A total of 107 Tera, Tera-CP, Scha and 2102EP cells were cultured for 24h after indicated treatment. Cells were harvested, washed with ice-cold PBS, and subsequently lysed in 500 µl lysis buffer (20 mM Tris HCl pH 7.6, 150 mM NaCl, 0.2% NP-40, protease inhibitor COMPLETE®, 1 mM PMSF, 1mM NaF and 1 mM DTT) for 15 minutes on ice. Cell lysates were clarified at 10,000g for 15 minutes; protein concentration was equalized with Bradford, and incubated for 16h with a mixture of agarose conjugated anti p53 (Do1 & FL-393, Santa Cruz, CA, USA). Immunocomplexes were washed 5 times and eluted with 0.5 M Glycine/HCl pH 2.4, mixed 1:1 with standard 2x Western Blot sample buffer and examined by Western blot analysis as described above.

SDS-Polyacrylamide Gel Electrophoresis and immunoblotting. 24h after indicated treatment cells were harvested and lysates were examined by WB as described previously (4,21). The following antibodies were used: mouse anti p53 (DO-1, Santa Cruz), mouse anti-Mdm2 (SMP14, Oncogene Research Products, Darmstadt, Germany), mouse anti β-Actin (MP Biomedicals, Eindhoven, the Netherlands), mouse anti p21 (F5, Santa Cruz), rabbit anti-Parp (Roche Diagnostics, Almere, the Netherlands), caspase 8 (1C12, Cell Signalling, MA, USA), and anti-FasL (C20, Santa Cruz). The antibody binding was eventually determined using horseradish peroxidase (HRP)-conjugated secondary antibodies (DAKO, Glostrup, Denmark) and visualised with the POD chemoluminescence kit (Roche Diagnostics). WB membranes were imaged with Molecular Imager Gel Doc XR System (Biorad, Veenendaal, the Netherlands). Equal protein loading was checked for with the Bradford total protein assay, Ponceau S staining of the blots and ß-actin immunobloting.

Apoptosis. Cells were continuously incubated with cisplatin for 24h at various concentrations. Acridine orange fluorescent staining of nuclei in unfixed cells was used to distinguish apoptotic from vital cells (4,21,48).

RNA interference. Small-interfering RNA (siRNA) specific for human p53, MDM2, FasL, and negative control (scrambled) were purchased from Eurogentec (Maastricht, the Netherlands). TC cells were transfected in 6 well plates with 5 µl of 20 µM siRNA duplex or siRNA antisense using Oligofectamine reagent according to the manufacturer's instructions (Invitrogen). After 24h, cells were treated with cisplatin. 24h after the treatment cells were harvested for protein isolation. Alternatively, in order to perform an apoptosis assay, at 24h after transfection, cell were harvested and plated in 96-well plate. The day after, cells were treated with cisplatin. Sequence for p53 I small interfering RNA (siRNA) molecules was 5'-GCA UGA ACC GGA GGC CCA UdTdT-3' (sense) and 5'-AUG GGC

CUC CGG UUC AUG CdTdT-3' (anti-sense), sequence for p53 II siRNA was 5'-CUU CGA CUU UGU CAC CGA GdTdT-3' (sense) and 5'-CUU ACG CUG AGU ACU UCG AdTdT-3' (anti-sense), sequence for P21 I siRNA was 5'-CUU CGA CUU UGU CAC CGA GdTdT-3' (sense) and 5'-CUU ACG CUG AGU ACU UCG AdTdT-3' (anti-sense), sequence for P21 II siRNA was 5'-GAC CAU GUG GAC CUG UCA CdTdT-3' (sense) and 5'-GUG ACA GGU CCA CAU GGU CdTdT-3' (antisense) , sequence for FasL siRNA was 5'-CTG GGC TGT ACT TTG TAT AdTdT-3' (sense) and 5'-TAT ACA AAG TAC AGC CCA GdTdT-3' (anti-sense).

Fas-membrane expression. TC cells were treated as indicated and eventually stained with a phycoerythrin (PE)-conjugated Ab against Fas (DX2, Becton Dickinson) for 1 hour at room temperature. Subsequently, cells were washed and analyzed by flow cytometry (FACS-Calibur; Becton Dickinson). The mean fluorescence intensity was determined by comparison of the fluorescence intensity of unlabeled cells.

Statistical Analysis. Results of at least 3 experiments are expressed as mean ± SD. Student's unpaired t test was used to compare values of test and control samples. All tests were 2-sided and differences were considered to indicate significance when $p < 0.05$

Results

P53 and MDM2 cellular localization and cisplatin response in TC cells. We used a panel of cisplatin-sensitive and -resistant wild type p53 expressing TC cell lines to compare cisplatin responses (Table 1) with the cellular localization of p53 and MDM2 and p53-MDM2 complex formation (Figure 1A-C, Supplemental Figure 1). Using immunofluorescence, we have observed that p53 predominantly localized to the cytoplasm, while MDM2 was mainly present in the nucleus in all four cell lines (Figure 1A & Supplemental Figure 1). Following exposure to 8 μM cisplatin, nuclear localization of p53 became more pronounced in cisplatin-sensitive cell lines Tera (Figure 1A) and 833KE (data not shown). In contrast, MDM2 expression was observed in both the nucleus and the cytoplasm of Tera cells upon cisplatin treatment (Figure 1A). In the intrinsically cisplatin-resistant TC cell lines, Scha and 2102EP, and in Tera-CP, an acquired cisplatin-resistant subline of Tera, sustained cytoplasmic p53 was observed at 8 μM cisplatin (Supplemental Figure 1). Only at higher cisplatin concentrations p53 became more nuclear localized (results not shown). In the cisplatin-resistant cell lines, MDM2 retained its nuclear localization after treatment with 8 μM cisplatin and became cytoplasmic only at high cisplatin concentrations. Immunoprecipitation (IP) was used to determine whether the observed shift in cellular localization of p53 and MDM2 after cisplatin treatment affected p53-MDM2 complex formation (Figure 1B). Note that we used lower cisplatin concentrations for Tera compared to the other cell lines. Despite the strong induction of both p53 and MDM2 in Tera with increasing concentrations of cisplatin (Figure 1C), IP experiments showed a decrease of p53-MDM2 complexes at cisplatin concentrations above 2 μM (Figure 1B). In cisplatin-resistant TC cell lines, we still observed sustained p53-MDM2 complexes even at relatively high (up to 8 μM) cisplatin concentrations. Eventually, p53-MDM2 complexes in these cells were partially lost at cisplatin concentrations above 16 μM (Figure 1B). Taken together, our results suggest that high sensitivity for cisplatin cytotoxicity and cisplatin-induced apoptosis is related to loss of p53-MDM2 complex formation and a change in p53 cellular localization.

Apoptosis induction by inhibition of the p53-MDM2 interactions depends on wild-type p53. To investigate the importance of p53-MDM2 complex formation in preventing apoptosis in TC, we used the small molecule inhibitors RITA and Nutlin-3 to inhibit the p53-MDM2 interaction. RITA induced massive apoptosis at nanomolar concentrations in the absence of transcriptional activation of any of the p53 targets tested (p53, MDM2, p21 and

Fas). Furthermore, downregulation of p53 with siRNA did not interfere with apoptosis induction by RITA (Supplemental Fig 2A). Moreover, treatment with RITA induced caspase-dependent apoptosis in the TC cell line NCCIT expressing mutant p53 (Supplemental Fig 2A). These results indicate that, at least in TC cells, RITA induces apoptosis independently of wild-type p53. We therefore focused on the effects of the small molecule inhibitor Nutlin-3 in wild-type p53 expressing TC cells.

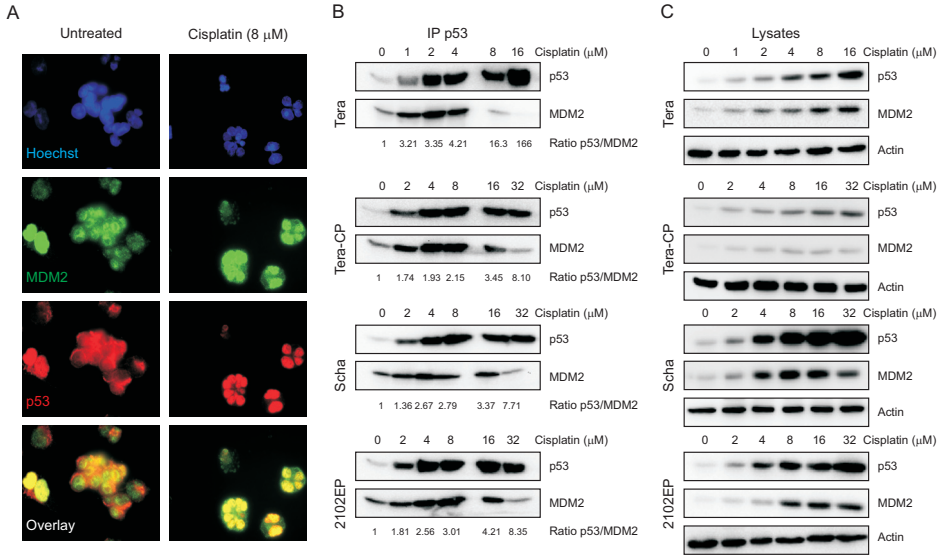

Figure 1. Loss of complex formation between p53 and MDM2, in cisplatin-sensitive TC cells (A) Immunofluorescence showing that p53 localizes in the nucleus, whereas MDM2 resides in the cytoplasm after cisplatin treatment in the cisplatin-sensitive TC cell line Tera, representative example of three independent experiments. (B-C) Note that we used lower cisplatin concentrations for Tera compared to the other TC cell lines. TC cells were harvested 12h after indicated cisplatin treatment. (B) Cell lysates were subjected to p53 immunoprecipitation (IP). Immunoblotting was performed using anti-p53 and anti-MDM2 antibodies. In the cisplatin-resistant TC cell lines Tera-CP, 2102EP and Scha, p53 was maintained in a complex with MDM2 after cisplatin treatment, while the cisplatin-sensitive Tera cells showed a loss of p53-MDM2 complex formation at low cisplatin concentrations. Relative levels of p53 and MDM2 were calculated with image J1.41 (National Institutes of Health) normalized and divided p53/MDM2. (C) Immunoblotting showing levels of p53, MDM2 and actin from lysates used for IP as indicated.

Treatment of cisplatin-sensitive Tera and 833KE or cisplatin-resistant Tera-CP, Scha and 2102EP cells, all expressing wild-type p53, with Nutlin-3 resulted in a dose-dependent reduction in cell survival, while no effect of Nutlin-3 on survival of NCCIT cells, that express a p53 mutant, was observed (Figure 2A). The reduced survival after Nutlin-3 treatment was caused by a dose-dependent induction of apoptosis, which is caspase-dependent as shown by increased PARP cleavage (Figure 2B-C). Although Nutlin-3 induced apoptosis in all wild-type p53 cell lines, low dose Nutlin-3 treatment (4 μM) led to a less prominent apoptotic response in the cisplatin-resistant compared to the cisplatin-sensitive cell lines (Figure 2B). Immunoblotting demonstrated specific upregulation of p53 after treatment of the various cell lines with Nutlin-3 and a dose-dependent increase in the expression levels of the p53 transcriptional targets MDM2 and p21, suggesting that p53 is transcriptionally active after

Nutlin-3 treatment (Figure 2C). Furthermore, Nutlin-3 treatment led to a more pronounced nuclear localization of p53 in all p53 wild-type cells (Supplemental Figure 2B). The mutant p53 NCCIT cell line was unaffected after treatment with Nutlin-3, further demonstrating the wild-type p53-dependent effect of Nutlin-3 (Figure 2B-C).

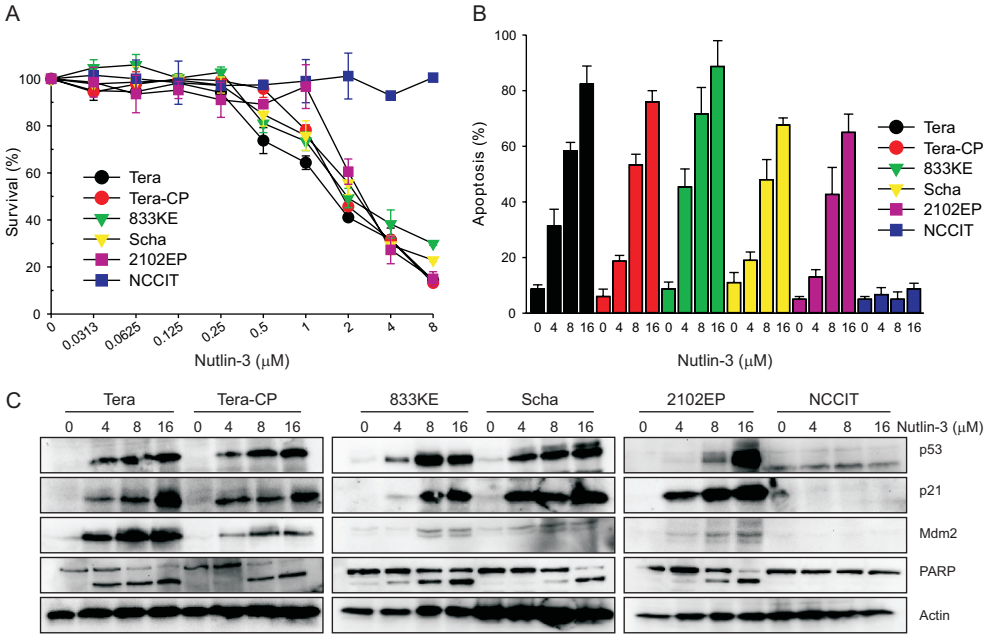

Figure 2. Nutlin-3 induced apoptosis in TC cells (A) Survival of TC cells after 96h of continuous Nutlin-3 treatment as indicated; values are the mean ± SD of three independent experiments. (B) Apoptosis induction was analyzed after Nutlin-3 treatment for 24h, by fluorescence microscopy on acridine orange stained TC cells; values are the mean ± SD of three experiments. (C) Immunoblot analysis showing upregulation of p53, MDM2 and p21 and enhanced cleavage of PARP and caspase-8 in wild-type p53 TC cells 24h after Nutlin-3 treatment. The data presented are representative of three independent experiments.

We further proved that the observed transcriptional activity and apoptosis induction after Nutlin-3 treatment was p53-dependent, since p53 downregulation by siRNA, prevented the upregulation of MDM2 and p21 levels after Nutlin-3 treatment (Figure 3D). In addition, p53 siRNA transfection, reduced the apoptotic response compared to scrambled siRNA after Nutlin-3 treatment in all wild-type p53 expressing cells (Figure 3A-C & Supplemental Figure 3A).

Increased Fas death receptor expression after Nutlin-3 treatment in TC cells. In line with our previous results (4), we found elevated Fas membrane expression levels after cisplatin treatment in Tera cells, less in Tera-CP cells and only minor changes in 2102EP and Scha cells (Figure 4A,B). Interestingly, Nutlin-3 treatment led to a robust upregulation of Fas membrane expression in all wild-type p53 expressing TC cell lines to a level significantly higher than solvent or cisplatin treatment (Figure 4A,B). Fas is induced in a p53-dependent manner in TC cells (4,8,21,49-52), which is further supported by our observation that no induction of Fas membrane expression was detected in the mutant p53 expressing TC cell line

NCCIT after either cisplatin or Nutlin-3 treatment (Supplemental Figure 3B). Additionally, Nutlin-3 treatment of p53-suppressed wild-type p53 TC cells prevented the induction of Fas membrane expression (Figure 4C). These results indicate that the observed upregulation of Fas membrane expression after Nutlin-3 treatment is wild-type p53 dependent as well. Previously, we have shown that cisplatin-induced apoptosis is depending on activation of the Fas/FasL system in the cisplatin-sensitive Tera and 833KE cells, while activation of the Fas/FasL system is inhibited in the cisplatin-resistant Tera-CP and Scha cells (4). We observed a significantly higher Fas membrane expression in all wild-type p53 cell lines, compared to solvent or cisplatin, suggesting the involvement of the Fas death receptor pathway in apoptosis-induction by Nutlin-3.

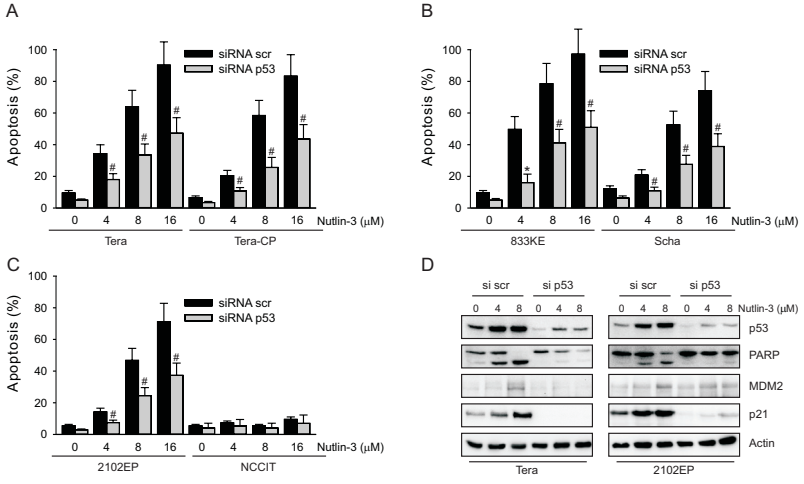

Figure 3. Wild-type p53 is necessary for the Nutlin-3 response (A-C) Downregulation of p53 reduced the apoptotic response after Nutlin-3 treatment in wild-type p53 expressing TC cells. Cells were treated with scrambled siRNA (siRNA scr) or with p53 siRNA (siRNA p53) for 24h. Values are the mean ± SD of three experiments; #$p < 0.05$; *$p < 0.01$; **$p < 0.005$. (D) Successful downregulation of p53 using siRNA, decreased the expression of MDM2 and p21, and decreased cleavage of PARP in p53-suppressed Nutlin-3 treated TC cells compared to control; a representative example of three independent experiments is shown.

Fas-dependent apoptosis after Nutlin-3 treatment. To further investigate the importance of Fas up-regulation for Nutlin-3-induced apoptosis, we inhibited the Fas/FasL interaction by either FasL blocking with Nok-1 antibody or using siRNA targeting FasL. Both blocking of FasL and suppression of FasL dramatically reduced the apoptotic response after high doses Nutlin-3 in all TC cell lines (Figure 4D), as visualized by a significant decrease in active, cleaved caspase-8 as well as a decrease in PARP cleavage (Figure 4E-F). Downregulation of FasL with siRNA was confirmed by immunoblotting (Figure 4E-F & Supplemental Figure 3C). Blocking or suppression of FasL, however, had no effect on the induction of p53 by Nutlin-3 (Figure 4E-F & Supplemental Figure 3C). These results indicate that the massive apoptosis induction in TC cells after Nutlin-3 treatment is to a large extent dependent on activation of the Fas death receptor pathway.

We recently established an important role for high cytoplasmic levels of p21 in inhibiting cisplatin-induced Fas-mediated apoptosis in TC (Spierings et al. 2004; Koster et al. 2010). Because of the reduced apoptotic response at the lowest Nutlin-3 dose (4μM) in the intrin-

sically cisplatin-resistant Scha and 2102EP cells, expressing high p21 levels, compared to the cisplatin-sensitive Tera and 833KE cells, expressing low levels of p21, (Figure 2B) we defined the importance of p21 in inhibiting Nutlin-3-induced apoptosis. Down-regulation of p21 using an siRNA approach (Supplemental Figure 3D) led to an increase in Nutlin-3-induced apoptosis 3 in Scha and 2102EP cells, as demonstrated by an increase in PARP cleavage (Supplemental Figure 3D). We, thus, show that p21 plays a role in inhibiting Nutlin-3 induced apoptosis as well, but only after treatment with relatively low Nutlin-3 concentrations.

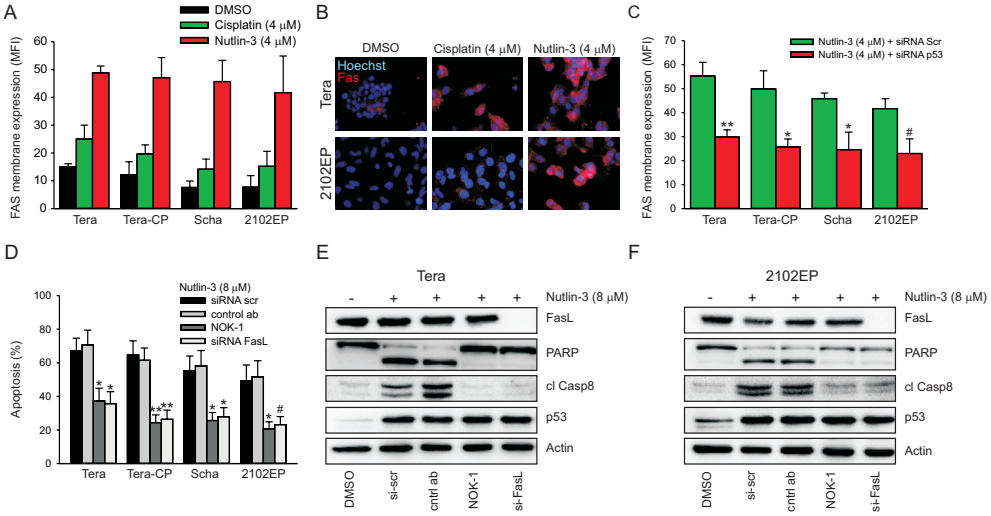

Figure 4. Fas-dependent apoptosis after Nutlin-3 treatment in TC cells (A) Following the indicated treatment, TC cells were harvested and Fas membrane expression was determined by flow cytometry. Values were depicted as mean fluorescence intensity (MFI). Values are the mean ± SD of three experiments. (B) TC cells were treated as indicated and Fas-membrane expression was determined by immunofluorescence. (C) Downregulation of p53 reduced Fas membrane expression after Nutlin-3 treatment in wild-type p53 expressing TC cells, compared to control; values are the mean ± SD of three experiments; #$p < 0.05$; *$p < 0.01$; **$p < 0.005$. (D) Decreased apoptotic response after blocking or suppression of FasL, in TC cells treated with Nutlin-3; values are the mean ± SD of three experiments. (E-F) Fas acts pro-apoptotic after 24h Nutlin-3 treatment in TC cells. After successful downregulation of FasL or blocking of FasL, with NOK-1, a decrease in PARP cleavage and active caspase-8 was observed in Tera (E) and 2102EP (F); a representative example of three independent experiments is shown.

Next, we addressed the question, if the Fas death receptor pathway also plays an important role in Nutlin-3-induced apoptosis in other, non-testicular tumour cell types. To this end, we analyzed Hodgkin lymphoma and AML cell lines, which are also known to be sensitive to Nutlin-3 (41,42,53). Blocking of FasL with Nok-1 considerably reduced the apoptotic response and PARP cleavage after Nutlin-3 treatment in the wild-type p53 expressing Hodgkin lymphoma cell lines KM-H2 and L540 (Figure 5A) as well as in the wild-type p53 expressing MOLM-13 AML cells (Figure 5B). In addition, no effect of either Nutlin-3 treatment or blocking of the Fas/FasL interaction was observed in mutant p53 Hodgkin lymphoma and AML cell lines, L428 (Figure 5A) and HL-60 (Figure 5B), respectively. These results indicate that the Fas death receptor pathway also plays an important role in Nutlin-3 induced apoptosis in wild-type p53 cell lines derived from other tumour types.

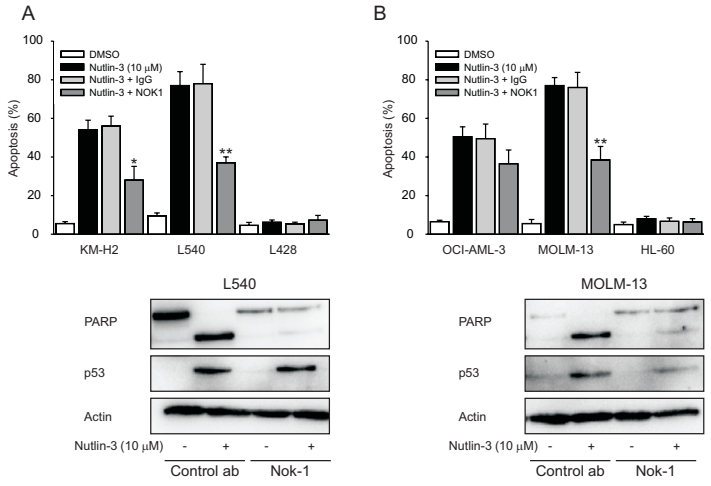

Figure 5. Fas-dependent apoptosis after Nutlin-3 treatment in Haematological Neoplasm (A-B) Decreased apoptotic response and decreased PARP cleavage after blocking of FasL in wild-type p53 expressing Hodgkin lymphoma cells (A) and AML cells (B) after Nutlin-3 treatment. Values are the mean ± SD of three experiments; #p < 0.05; *p < 0.01; **p < 0.005. A representative example of three independent experiments is shown.

Synergistic effect of Nutlin-3 and cisplatin is Fas death receptor- and p53-dependent. Finally, we tested the cytotoxicity of cisplatin in combination with minimally toxic concentrations of Nutlin-3 in TC cells. Treatment for 96h with the combined drugs led to much stronger reductions in survival of all wild-type p53 TC cell lines tested as compared to single drug treatment with cisplatin or Nutlin-3 (Figure 6A, Supplemental Figure 4A). The strongest decrease in survival combining the two drugs was observed in the intrinsic and acquired cisplatin-resistant cell lines (Figure 6A, Supplemental Figure 4A & Table 1). As expected, the combination with Nutlin-3 had no potentiating effect on cisplatin-induced cytotoxicity in the mutant p53 cell line NCCIT (Supplemental Figure 4B).

The reduction in survival of TC cells after the combined treatment with Nutlin-3 and cisplatin was related to the enhanced induction of apoptosis (Figure 6C & Supplemental Figure 6B). Immunoblotting showed enhanced upregulation of p53 and MDM2 after combined treatment with Nutlin-3 and cisplatin compared to either cisplatin or Nutlin-3 treatment alone (Figure 6B & Supplemental Figure 6A). The increase in PARP cleavage demonstrated the enhanced activation of effector caspases by the combined treatment (Figure 6B & Supplemental Figure 6A). Next we addressed the question, if the Fas death receptor pathway also plays an important role in apoptosis-induction after the combined treatment. Therefore, we first determined Fas membrane expression levels after the combined treatment. Fas membrane expression was in the combination with cisplatin, compared to Nutlin-3 alone, even to a greater extent upregulated in all wild-type p53 TC cell lines (Figure 6D & Supplemental Figure 5C), while no induction of Fas membrane expression was observed in the mutant p53 expressing NCCIT (Supplemental Figure 3B). Additionally, blocking of FasL reduced the apoptotic response of wild-type p53 TC cells to the combined treatment with Nutlin-3 and cisplatin, in particular at the lower cisplatin concentration (Figure 6E & Supplemental Figure 5D). Suppression of MDM2 with siRNA extremely sensitized TC cells to cisplatin treatment, almost similar to our observations with Nutlin-3. This demonstrates that the release of the negative feedback on p53 by MDM2 is the important event in the induction of apoptosis in TC cells (Figure 6B-C). Moreover, p53 expression and Fas membrane expression were strongly upregulated after treatment with MDM2 siRNA, especially in combination with cisplatin in the cisplatin-resistant cells (Figure 6B,D & Supplemental Figure 5A,C).

These observations indicate that targeting MDM2 in combination with cisplatin treatment might potentially overcome both intrinsic as well as acquired-resistance to cisplatin in wild-type p53 TC, and is largely dependent on activation of the Fas death receptor pathway.

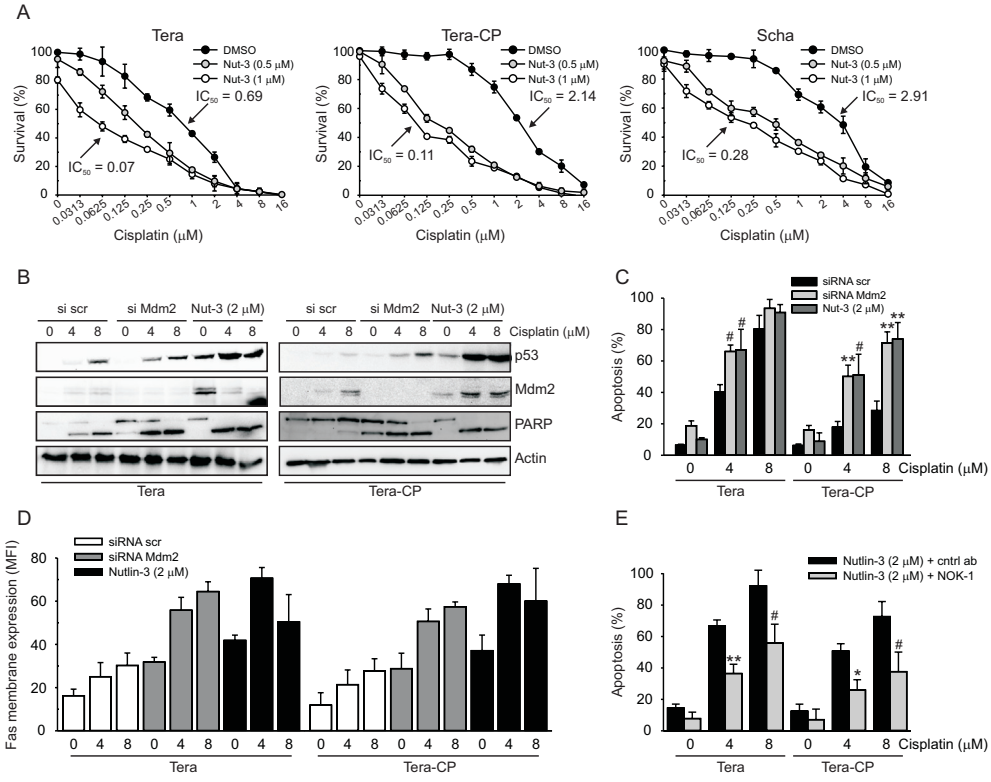

Figure 6. Synergistic effect of cisplatin and inhibition of the p53-MDM2 interaction (A) Survival of TC cells after 96h of continuous Nutlin-3 (Nut-3) treatment as indicated, in combination with increasing cisplatin concentration. IC_{50} values are depicted for cisplatin as well as the combination with 1 μM Nutlin-3; values are the mean ± SD. (B) Increased levels of p53 and increased PARP cleavage after targeting the p53/MDM2 axis; a representative example of three independent experiments is shown. (C) Increased apoptosis after targeting the p53/MDM2 axis with either siRNA against MDM2 or Nutlin-3 (Nut-3) in combination with cisplatin; values are the mean ± SD of three experiments; #$p < 0.05$; *$p < 0.01$; **$p < 0.005$. (D) Following indicated treatment TC cells were harvested and Fas membrane expression was determined by flow cytometry. Values were depicted as mean fluorescence intensity (MFI). Values are the mean ± SD of three experiments (E) Decreased apoptotic response after blocking of FasL, with NOK-1, in TC cells treated with the combination of cisplatin and Nutlin-3 as indicated; values are the mean ± SD of three experiments; #$p < 0.05$; *$p < 0.01$; **$p < 0.005$.

Discussion

In the present study, we demonstrate that wild-type p53 is sustained in complex with MDM2 in cisplatin-resistant TC cell lines following treatment with therapeutically-relevant cisplatin concentrations. Our results indicate that interfering in the p53-MDM2 interaction through the small molecule compound Nutlin-3 sensitizes wild-type p53 TC cells for apoptosis. Combining Nutlin-3 with cisplatin, the most important therapeutic drug in the treatment of testicular

cancer patients, results in hyper-activation of the p53 pathway and largely sensitizes both intrinsic as well as acquired cisplatin-resistant TC cells to apoptosis via the Fas/FasL death receptor pathway and strongly reduces cell survival. These results indicate that targeting the p53/MDM2 axis, in combination with standard chemotherapeutic treatment, appears to be an attractive therapeutic strategy to pursue for cisplatin-resistant/refractory testicular cancer (Figure 7).

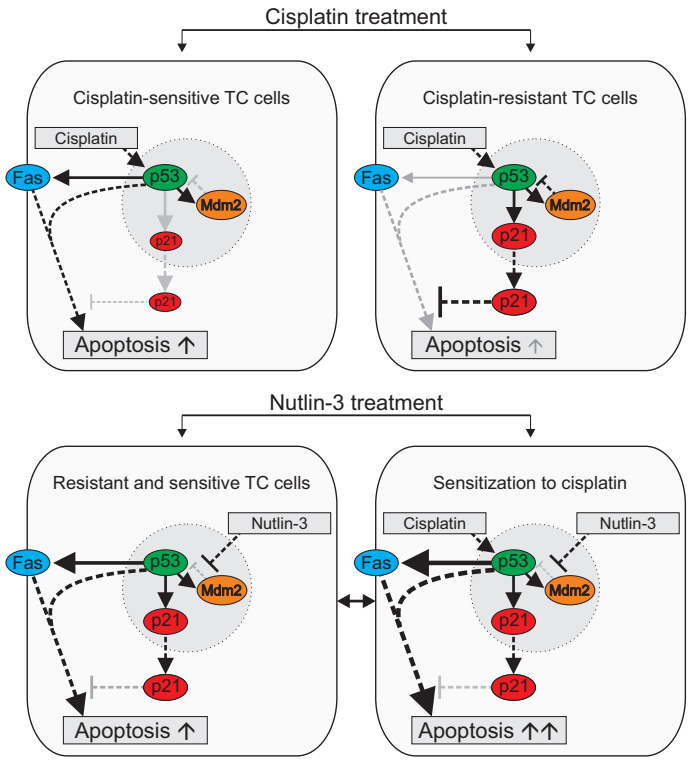

Figure 7. Simplified model showing the role of the MDM2/p53 axis in regulating the sensitivity to cisplatin and Nutlin-3 in wild-type p53 expressing TC cells. P53 is a transcriptional activator of p21, Fas and MDM2. Cisplatin sensitive TC cells have low levels of p21 and cisplatin-resistant TC cells high levels of cytoplasmic p21, which is a key determinant of cisplatin-induced apoptosis (36). Cisplatin-induced apoptosis in TC cells also involves activation of the Fas death receptor pathway via elevated Fas membrane expression. High cytoplasmic p21 levels inhibit Fas death receptor mediated apoptosis in cisplatin-resistant TC cells (21). Moreover, cisplatin-induced DNA damage activates p53 and enhances release of p53 from p53-MDM2 complex, while sustained p53-MDM2 complex formation is found in cisplatin resistant cells. Interfering in p53-MDM2 complex formation by Nutlin-3 treatment (or suppression of MDM2) substantially induced Fas expression, resulting in apoptosis of both cisplatin sensitive and resistant TC cells. Cisplatin in combination with Nutlin-3 further enhanced Fas expression and sensitized cisplatin-sensitive and resistant TC cells to cisplatin-induced apoptosis.

P53-interacting proteins, such as MDM2, are important regulators of wild-type p53 functionality (39,54-60). The lack of *TP53* mutations in TC led to the hypothesis that constitutively expressed p53 is functionally inactive (61-63). Surprisingly high levels of wild-type p53 have been frequently observed in TC that are correlated with expression levels of the p53 transcriptional target MDM2, suggesting that p53 is functional in TC (20,26,29,34,64). Here, we show that treatment with the selective MDM2 antagonist Nutlin-3 caused a high induction of both p53 and MDM2, a massive induction of apoptosis, and a strong reduction in cell survival in cisplatin-sensitive as well as cisplatin-resistant TC cell lines. In addition, siRNA targeting MDM2 had similar effects as Nutlin-3 treatment, indicating an important role of MDM2 as a negative-feedback regulator of p53 activity, and excluding a role for Nutlin-3

induced release of MDM2 in the observed apoptosis. These results suggest a tight regulation of p53 interacting with MDM2. Several in vitro and in vivo studies suggest an important role for p53 in TC cisplatin responses (26,31,63,65-67). In contrast, other studies have failed to support a role for p53 in TC responses (29), although the involvement of the p53-MDM2 complex in the response to chemotherapy had not been thoroughly assessed (29,30). The present study indicates that p53 function is impeded by the interaction with MDM2 and the subsequent sequestration of p53 in the cytoplasm of TC cells following cisplatin treatment. The strong sensitization to cisplatin by low doses of Nutlin-3 further demonstrates the importance of MDM2 in controlling p53 following DNA damage.

Table1 – IC_{50} values and p53 status of the cell lines used in this study

Cell line	IC_{50} Cisplatin (μM)[1]	IC_{50} Combination (μM)[2]	Enhancement Ratio[3]	P53 status[4]
Tera	0,69 ± 0,11	0,07 ± 0,01	9,9	wt/wt
Tera-CP	2,14 ± 0,17	0,11 ± 0,02	19,5	wt/wt
833KE	1,04 ± 0,10	0,13 ± 0,03	8,0	wt/wt
Scha	2,91 ± 0,84	0,28 ± 0,08	10,4	wt/wt
2102EP	4,05 ± 0,66	0,39 ± 0,21	10,4	wt/wt
NCCIT	2,15 ± 0,34	2,25 ± 0,59	1,0	mt/-

1) The IC_{50} (drug concentration reducing cell survival by 50%) for cisplatin was calculated from the graphs in Figure 6A and Supplemental Figure 6A-B. The mean IC_{50} ± SD was determined in three experiments, each performed in quadruplicate.

2) The IC_{50} (drug concentration reducing cell survival by 50%) for cisplatin in combination with 1 μM Nutlin-3 was calculated from the graphs in Figure 6A and Supplemental Figure 6A-B. The mean IC_{50} ± SD was determined in three experiments, each performed in quadruplicate.

3) Nutlin-3 enhancement ratios were calculated by dividing the IC_{50} for cisplatin alone by the IC_{50} for the combination with cisplatin and 1 μM Nutlin-3.

4) p53 status of the cells was previously described (21,24).

Induction of FasL and upregulation of the Fas receptor in a p53-dependent manner has been observed in several tumour cell lines after treatment with chemotherapeutic drugs, such as cisplatin, and is related to apoptosis induction (4,8,21,49-52). Previously, we have reported that the Fas/FasL system is active and functional in cisplatin-sensitive but almost inactive in cisplatin-resistant TC cell lines (4). In the present study, we show that Nutlin-3 treatment strongly enhanced Fas membrane expression levels in TC cells. Fascinatingly, blocking of the Fas/FasL death receptor pathway in TC cells impairs apoptosis induction by Nutlin-3 as well as combined treatment with Nutlin-3 and cisplatin. However, involvement of other genes in this pathway cannot be excluded, because p53 has been shown to transcriptionally activate several other genes involved in apoptosis via the Fas/FasL system after cisplatin treatment. In the cisplatin-sensitive Tera cells, besides *FAS*, the Fas adaptor *LRDD* and a gene implicated in positive Fas regulation, *PHLDA3* were found to be regulated by p53 upon cisplatin treatment (31). Importantly, we observed that the Fas/FasL death receptor pathway also plays an important role in Nutlin-3-induced apoptosis of wild-type p53-expressing AML and Hodgkin cell lines. Interestingly, the wild-type p53-expressing cell line OCI-AML-3 did not show Nutlin-3-induced apoptosis. These latter results, however, can be explained by observations that Nutlin-3-induced apoptosis was independent of transcriptional activation of p53 in these cells (42). These results indicate a major role for the Fas/FasL death receptor

pathway in response to Nutlin-3 in wild-type p53 TC, AML and Hodgkin cell lines.

Recently, we have defined the important protective role of high, cytoplasmic localized p21 levels against cisplatin-induced Fas receptor-mediated apoptosis in cisplatin-resistant TC cells (21,36). The present results indicate that p21 only blocks Nutlin-3-induced apoptosis in cisplatin-resistant TC cells at relatively low Nutlin-3 concentrations. Treatment with higher Nutlin-3 concentrations led to a strong induction of p53 and a further increase in Fas membrane expression in testicular cancer cells, whereas p21 mRNA and protein levels remained relatively low after either Nutlin-3 or cisplatin treatment in these cells (4,21,36,44). Non-apoptotic genes, such as *CDKN1A*, constitutively harbour high levels of the poised RNA polymerase II (RNAPII) initiation complex at their core promoters, which are converted into elongated forms shortly after stress, but reinitiate very poorly. In contrast, pro-apoptotic genes, including *FAS*, have low levels of bound RNAPII but undergo damage-induced activation through multiple rounds of efficient reinitiation (68-70). Additionally, the co-factors ASPP1/2, JMY, HAUSP, and NF-Y enhance p53 apoptotic activity by facilitating its binding to pro-apoptotic promoters (69-73). For instance, NF-Y is known to be an essential positive regulator of *FAS* transcription, while it represses *CDKN1A* promoter activity (69,70). Interestingly, p53 occupancy of the p21 promoter in testicular cancer cells was similar to the levels observed in U2OS and HCT116 cells, whereas much less p21 expression was found in testicular cancer cells (44). Therefore, it is tempting to speculate that the Nutlin-3 induced release of the negative feedback on p53 by MDM2 further shifts the balance toward transcribing pro-apoptotic genes (involved in the Fas death receptor pathway).

Combining Nutlin-3 with other cytotoxic agents enhanced the activity of these agents in wild-type p53 leukaemia cells (47,74,75). Our results showed that combining non-toxic concentrations of Nutlin-3 with cisplatin sensitized both intrinsic as well as acquired cisplatin-resistant TC cells to low concentrations of cisplatin. Of interest, MDM2 inhibitors in contrast to cisplatin might be considered non-genotoxic, as demonstrated with Nutlin-3a in mice (76). Nutlin-3a has even shown protective activity in normal kidney cells against cisplatin-induced apoptosis (77). MDM2 inhibitors are in phase I clinical trials in haematological malignancies and solid tumours. Based on the present results, clinical trials using MDM2 inhibitors such as Nutlin-3 in combination with cisplatin to treat cancer patients with wild-type p53, for instance refractory TC patients, may be of great importance.

In conclusion, we demonstrate that the negative feedback regulator MDM2 plays an important role in the sensitivity of wild-type p53 testicular cancer to Nutlin-3 and cisplatin. These findings indicate that targeting the p53/MDM2 axis in combination with standard treatment enhances Fas death receptor-mediated apoptosis, is a therapeutic strategy warranted to pursue in cisplatin-resistant/refractory testicular cancers.

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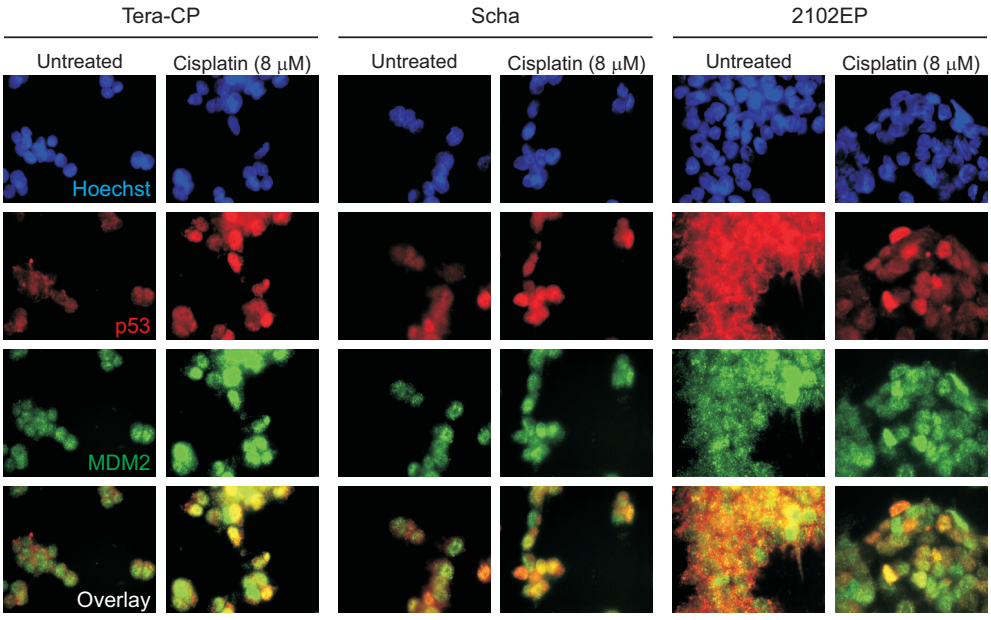
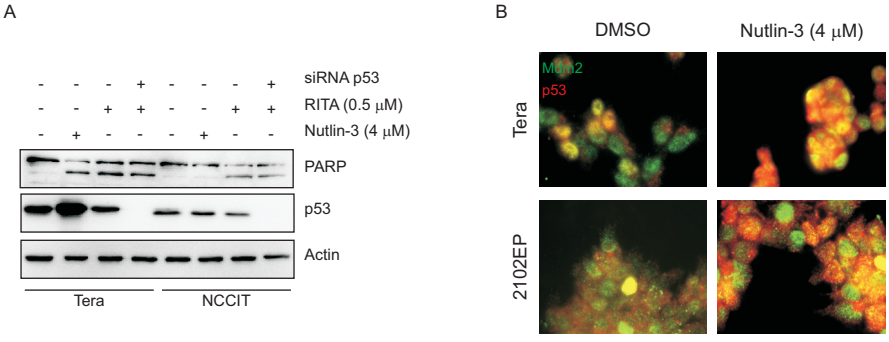
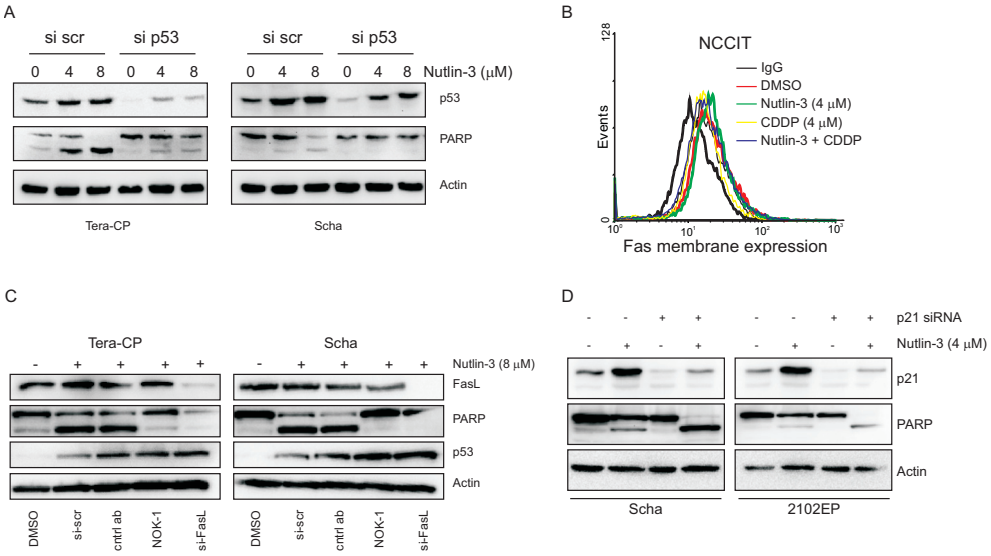


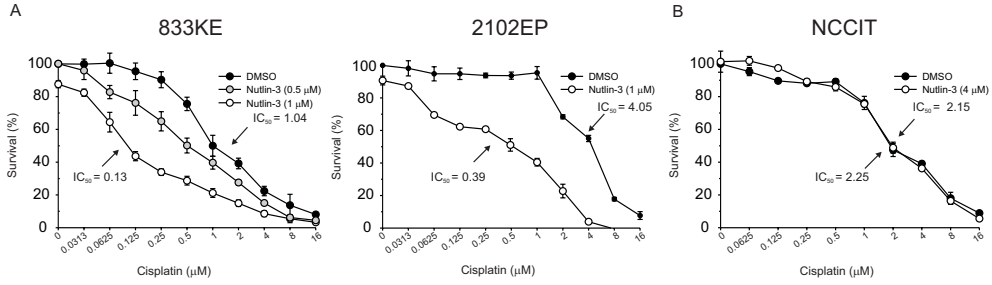
Supplemental Figure 1. Immunofluorescence showing that p53 becomes more pronounced nuclear localized, while MDM2 stays nuclear localized after cisplatin treatment (8 μM) in the cisplatin-resistant TC cell line Tera-CP, Scha and 2102EP; a representative example of three independent experiments is shown.



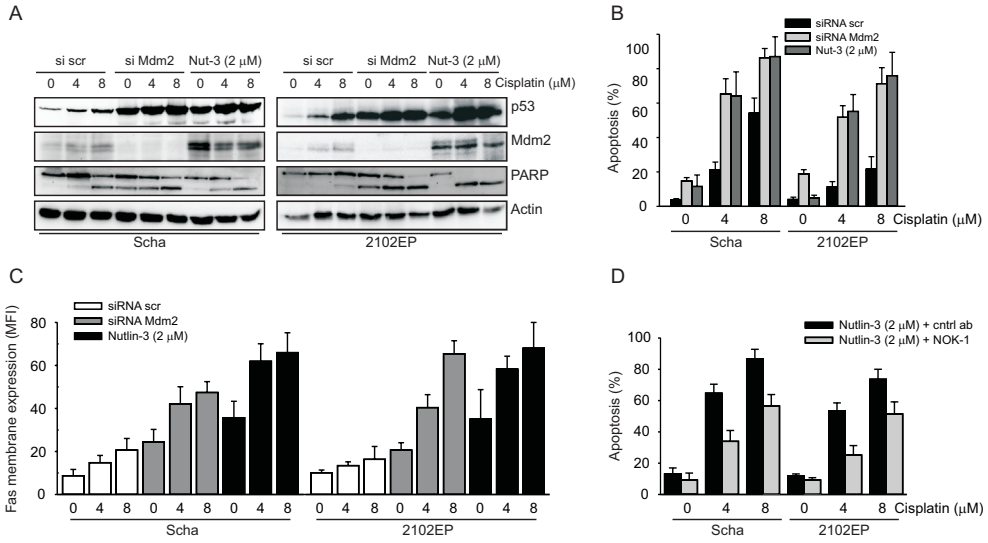
Supplemental Figure 2. (A) No induction of p53 after treatment with RITA. Suppression of p53 with a specific siRNA did not interfere with the apoptosis induction by RITA as visualised by PARP cleavage; a representative example of three independent experiments is shown. (B) Immunofluorescence showing that p53 is more nuclear localized after Nutlin-3 treatment in the cisplatin-sensitive TC cell line Tera and the intrinsically resistant cell line 2102EP compared to control; a representative example of three independent experiments is shown.



Supplemental Figure 3. (A) Successful downregulation of p53 using siRNA targeting p53, decreased cleavage of PARP in p53-suppressed Nutlin-3 treated TC cells compared to control; a representative example of three independent experiments is shown. (B) Following the indicated treatment no effect was observed on Fas membrane expression of the mutant p53 cell line NCCIT; a representative example of three independent experiments is shown. (C) After successful downregulation of FasL or blocking of FasL, with NOK-1, a decrease in PARP cleavage was observed in Tera-CP and Scha; a representative example of three independent experiments is shown. (D) Downregulation of p21 increased the apoptotic response of the intrinsically ciplatin-resistant cells Scha and 2102EP after Nutlin-3 treatment; a representative example of three independent experiments is shown.



Supplemental Figure 4. (A-B) Survival of TC cells after 96h of continuous Nutlin-3 treatment as indicated, in combination with increasing cisplatin concentration. IC_{50} values are depicted for cisplatin as well as the combination; values are the mean ± SD.



Supplemental Figure 5. (A) Increased levels of p53 and increased PARP cleavage after targeting the p53/MDM2 axis; a representative example of three independent experiments is shown. (B) Increased apoptosis after targeting the p53/MDM2 axis with either siRNA against MDM2 or Nutlin-3 (Nut-3) in combination with cisplatin; values are the mean ± SD of three experiments. (C) Following the indicated treatment TC cells were harvested and Fas membrane expression was determined by flow cytometry. Values were depicted as mean fluorescence intensity (MFI). Values are the mean ± SD of three experiments (D) Decreased apoptotic response after blocking of FasL, with NOK-1, in TC cells treated with the combination of cisplatin and Nutlin-3; Values are the mean ± SD of three experiments.

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Chapter 5

Testicular cancer a model for curable disease and a nursery for new cancer drug development

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and Steven de Jong

Manuscript in preparation

Abstract

Testicular cancers are the most frequent solid malignant tumor in men 20–40 years of age and the most frequent cause of death from solid tumors in this age group. At the time of diagnosis up to 50% of the patients suffer from metastatic disease, while the majority of metastatic testicular cancer patients, in contrast to most other metastatic solid tumors, can be cured with highly effective cisplatin-based chemotherapy. Therefore testicular cancer is considered the paradigm of curable solid tumor and an ideal model to investigate and unravel the molecular determinants of chemotherapy sensitivity (and resistance) of solid tumors. This review aims to summarize the current knowledge on the biological basis of cisplatin-induced apoptosis and response to chemotherapy in testicular cancer. Moreover, the fact that testicular cancer sustains high basal levels of wild-type p53 is discussed. In turn, presence of wild-type p53 is causative for the exquisite sensitivity to cisplatin of testicular cancers. Resistance to cisplatin occurs via a shift in the balance between key-players, such as $p21^{Waf1/Cip1}$ (p21), Fas and MDM2, acting upon cisplatin-induced activation of the p53-pathway. Recent studies suggest that high levels of cytoplasmic localized p21 and tight negative feedback regulation of p53 by MDM2 protect testicular cancer cells against cisplatin-induced apoptosis. Drugs targeting either cytoplasmic localized p21 or the p53/MDM2 axis showed, at least pre-clinically, a sensitizing effect to cisplatin treatment. Further clinical development of such type of drugs may lead to new treatment options for platinum resistant disease.

Introduction

Testicular cancers represent the most frequent solid malignant tumor in men 20-40 years of age and incidence is still rising world-wide (1). Based on histology, biochemical and clinical features these tumors can be divided in seminomatous and nonseminomatous testicular cancer. Non-seminomas do metastasize more widely and are less sensitive to radiotherapy than seminomatous testicular cancer, which has consequences for prognosis and treatment strategies. Nonseminomas display various stages of differentiation, ranging from the undifferentiated embryonal carcinoma (EC), the more differentiated extra-embryonic components like yolk sac carcinomas (YS) and choriocarcinomas (Chc), to the highly differentiated teratoma (T) component (2,3). Note, that most human cancer cell lines commonly used as model for testicular cancer are of EC origin.

Platinum-based chemotherapy is the cornerstone of treatment of many cancers and used as first-line therapy for testicular, bladder, lung, ovarian cancers. In case of bladder, lung and ovarian cancer there is often responsiveness to platinum-based chemotherapy, however the majority of these cancer patients will eventually relapse with platinum-resistant disease. In contrast, an enormous improvement has been obtained in outcome and survival of testicular cancer patients, even in case of extensive metastatic disease, by the introduction of cisplatin as anticancer drug in the mid 1970s (4,5). Testicular cancer is therefore considered as paradigm of platinum-sensitive solid tumors.

A relatively unique feature of testicular cancers is the expression of high levels of wild-type p53 (6-8) that is otherwise frequently mutated in other tumor types (9-12). Mutations in *TP53* that compromise p53 function occur in ~50% of human cancers (9-12). The mutation rate of p53 varies dramatically between different tumor types ranging from 10–12% in leukemias, to 38–70% in lung cancers and 43–60% in colon cancers, while in human testicular cancers surprisingly less than 1% of *TP53* mutations occur (13). *TP53* is a tumor suppressor gene, of which the protein has a dual role in stress response. It trans-activates a number of genes including $p21^{Waf1/Cip1}$ (p21), MDM2 and Fas (14) that coordinately direct cells into either cell cycle arrest or apoptosis. The p53 pathway is crucial for effective tumor suppression in humans and a major role for p53 in the response to chemotherapeutic drugs, such as cisplatin, and the execution of apoptosis has been described (15-19).

This review will briefly give an overview of common pathways activated by cisplatin-induced DNA-damage and thereafter mainly focuses on the functionality of wild-type p53, the p53-apoptotic pathway and downstream targets Fas, MDM2 and p21 and their role in determining the response to cisplatin-induced apoptosis in testicular cancer. Unfortunately, in the group of patients diagnosed with testicular cancer, a subset of patients with extensive metastatic spread belonging to the intermediate or poor risk group will not achieve a durable complete remission after initial treatment and eventually die from their disease (20). In depth understanding why most testicular cancer patients are curable with chemotherapy could lead to the development of more effective treatment of refractory testicular cancers and other less chemo-sensitive solid tumor types.

Response to cisplatin-induced DNA-damage. Cisplatin becomes activated intracellularly by the aquation of one or both of the two chloride groups before it covalently binds to DNA, forming DNA adducts (21). These adducts cause inter- and intrastrand DNA cross-links and distortions in DNA, including unwinding and bending, which are recognized by several cellular proteins (21,22). This process activates various signal-transduction pathways

involved in DNA-damage recognition (DDR) leading to cell-cycle arrest and DNA repair or apoptosis (21-23). DNA damage activates the ATM/ATR protein-kinase family. ATM/ATR in turn induces a range of differential posttranslational modifications of p53 (24-26), although conflicting data about the importance of these modifications, including p53 phosphorylation, have been published (27,28). P53 regulating proteins, such as the closely related proteins MDM2 and MDMX, have been suggested to be of great importance in tailoring the p53-mediated DNA damage response (27-31). ATM directly and indirectly induces MDM2 phosphorylation, resulting in decreased activity and stability of MDM2 (31-33) and, consequently, loss of the negative feedback on p53. Upon DNA damage induction, for example by cisplatin, p53 is activated and co-factors facilitate p53-mediated trans-activation of genes. Co-factors such as ASPP1/2, JMY, HAUSP and NF-Y seem to enhance p53 apoptotic activity by facilitating its binding to pro-apoptotic promoters (e.g. induction of Fas, TRAIL Death Receptor 5 and bax) (31,32,34-38), while co-factors such as c-abl, hnRNP K and Miz-1 facilitate cell cycle arrest, especially by enhancing p21 transcription, which allows time for DNA repair (34,39,40).

P21 was initially identified as an important mediator of p53-dependent tumor growth suppression, (41) functioning as an inhibitor of especially G1 cyclin kinases (42). P21 belongs to the Cip/Kip family of CDK inhibitors including p27 and p57. Each family member inhibits the kinase activity of broad but not identical classes of CDK–cyclin complexes, causing hypo-phosphorylation of retinoblastoma protein (pRb) (43,44). Hypo-phosphorylated pRb prevents the release of E2F (43-45), leading to the repression of E2F-dependent transcription and to a G0/G1 arrest (46,47). However, more recent investigations have shown that p21, besides inhibiting the cell cycle, might also play a role in promoting cell cycle transition (17,48-51), differentiation (52-54) and preventing apoptosis (55).

Lack of mutations in TP53 in TC. Aforementioned, a surprisingly low percentage of TP53 mutations are found in testicular cancer (TC). Lack of mutations in *TP53* might be explained by expression of microRNAs (miRs), which can inhibit translation of genes, including downstream targets of p53, through interaction with the 3'untranslated region (UTR) of the messenger RNA (mRNA). In TC, high expression of the miR-371~373 cluster was observed, which mimics mutated p53 by targeting the tumor suppressor gene protein LATS-2, involved in the regulation of G1-S transition in the cell cycle (56). Additionally, TCs are supposed to have a deregulated G1/S checkpoint (57-60), since p21 and pRB are almost not detectable in seminomas and embryonal carcinomas, while p27, cyclin E and cyclin D2 are over-expressed (57-63). Furthermore, p27 and p21 (if expressed) are mainly cytoplasmic localized in TC cell lines due to PI3K/Akt phosphorylation of the nuclear localization signal (NLS) domain of these proteins (64,65). Activated Akt TC might be due to the lack of PTEN (phosphatase and tensin homolog) (65) a negative regulator of the PI3K/Akt pathway. In turn, cytoplasmic p21 and p27 actually promote cell cycle progression from G1 into S phase, by regulating the assembly and activation of Cyclin/CDK complexes, a mechanism that has not been demonstrated in TC cells yet (17,48-51). Moreover, in TC, in contrast to other solid tumor types, the DDR machinery is rarely activated spontaneously (66,67). Therefore, no selective pressure on aberrations of the DDR genes, such as ATM/ATR, Chk2 and TP53 are necessary, whilst the intact state of the DDR machinery may contribute to the exquisite cisplatin-sensitivity of these tumors (66,67).

The role of wild type p53 in cisplatin-induced DNA damage and apoptosis in TC. High levels of wild-type p53 are positively correlated with MDM2 expression levels in vivo and in vitro in TC (6,62,68-70). MDM2 is an important negative feedback regulator of p53 (27-31), and activity towards p53 is regulated by phosphorylation of MDM2 (31,33,71,72). p-Akt can bind to and phosphorylate MDM2, leading to nuclear localization of MDM2 and consequently enhancing MDM2-mediated ubiquitination and degradation of p53 (71,72). Alternatively, p-ATM mediated phosporylation of MDM2 inhibits the ability of MDM2 to poly-ubiquitinate p53, and is likely to be the key step in causing p53 stabilization (31,33). Therefore, tight regulation of p53 by MDM2 may be of importance for the response to cisplatin in TC. In contrast to many other tumor cell types, in TC cell lines cisplatin treatment led to an upregulation of p53, MDM2 and Fas, while levels of p21 were much less affected Chapters 1-4 & refs (7,73). Moreover, we showed that cisplatin-induced apoptosis as well as trans-activation of MDM2, Fas en p21 were depending on p53 (Chapter 3). In cisplatin-sensitive TC cells, p53 downregulation strongly reduced sensitivity to cisplatin (Chapter 3). Interestingly, previous results from our group have demonstrated that cisplatin induces apoptosis mainly in as Fas-dependent manner in cisplatin-sensitive TC cells (73). In the cisplatin-sensitive Tera cells, besides *FAS*, the Fas adaptor *LRDD* and a gene implicated in positive Fas regulation, *PHLDA3* were found to be regulated by p53 upon cisplatin treatment (74). The pro-apoptotic role of CDK2 in TC has been recently established and is strongly connected to the Fas apoptosis route (64). CDK2 can be induced in a caspase-dependent way following activation of the Fas pathway (75), leading to depolarization of the mitochondrial membrane (76) by cytoplasmic CDK2 mediated translocation of Bax to the mitochondria (76,77). Alternatively, after the induction of DNA damage, functional interaction of CDK2 with FOXO1 can be an important mechanism to enhance, among others Fas ligand expression, and thus promotes activation of the Fas pathway (78). In contrast to the protective effect of p53 downregulation on cisplatin-induced apoptosis observed in cisplatin-sensitive TC cells, p53 suppression strongly sensitized intrinsic cisplatin-resistant TC cells to cisplatin (Chapter 3). The anti-apoptotic role of p53 may be due to its role as transcriptional activator of p21, a protein that was predominantly expressed in the intrinsic resistant TC cells (Chapter 3). In addition, in cisplatin-resistant TC cells p53 function is impeded by the interaction with MDM2 and the subsequent sequestration of p53 in the cytoplasm (Chapter 4). The importance of p21 and MDM2 in cisplatin-resistance of TC cells is discussed hereafter.

Cytoplasmic p21 as an inhibitor of cisplatin-induced apoptosis in TC. Recent studies on cisplatin and irradiation induced p21 in TC cell lines showed that irradiation, in contrast to cisplatin, strongly induced p21 mRNA and protein expression in these cells. Surprisingly, p21 was not present in the nucleus but in the cytoplasm. Functional analysis of irradiation-induced cytoplasmic p21 revealed its involvement in protecting TC cells against Fas-induced apoptosis (7). Whether differences in basal p21 levels are important in determining cisplatin sensitivity in TC cell lines, was, however, not studied. We have now reported that in cisplatin-sensitive TC cells lower basal levels of cytoplasmic localized p21 were detectable compared to intrinsically cisplatin-resistant TC cells (64). Moreover, ectopic expression of cytoplasmic localized p21, using a p21-Δ-NLS construct, rendered cisplatin-sensitive Tera cells resistant to cisplatin treatment. While p21 suppression of intrinsic cisplatin-resistant TC cells led to an increase in apoptosis upon cisplatin treatment (64). Clinical relevance of these cell line based findings was also demonstrated. As stated before, p21 is not commonly ex-

pressed in EC components of chemo-sensitive TC patients. In contrast, cytoplasmic localized p21 is detectable in the EC component of the majority of refractory testicular cancer patients suggesting a role for cytoplasmic p21 in the chemo-resistance of testicular cancers, probably via apoptosis inhibition. In two p21 positive, refractory TC patients only the EC component was present, which is further strengthening the in vitro observations with respect to the cytoplasmic p21 dependent mechanism of cisplatin resistance (64).

Cytoplasmic p21 antagonizes the pro-apoptotic function of proteins. The mechanism by which cytoplasmic p21 can inhibit apoptosis in TC is becoming more clear. Several possible anti-apoptotic functions of high cytoplasmic p21 levels have been described that are strongly related to Fas-induced apoptosis. ASK-1 is a mediator of Fas-induced apoptosis: upon Fas activation, Fas is trimerized and induces recruitment of Daxx that interacts with ASK1, resulting in activation of JNK/SAPK and p38, and concomitantly apoptosis induction. This process is blocked by cytoplasmic p21 binding to ASK1 (79-81). In fact, we have recently demonstrated that cytoplasmic p21 forms a complex with ASK-1 in TC (64). Additionally, cytoplasmic p21 can inhibit Rho-kinase activity (82,83), which in turn is involved in Fas-induced apoptosis by enhancing Fas/FasL clustering in lipid rafts and Fas receptor capping, a mechanism that has not been demonstrated in TC cells yet (84-86). Interaction of cytoplasmic localized p21 with caspase 3 can lead to reduced Fas-induced apoptosis, however in TC cells the interaction between cytoplasmic p21 and caspase 3 was not detected (7,64,87). CDK2 activity can be induced in a caspase-dependent way following activation of the Fas pathway (75). We have reported that CDK2 was in complex with p21 in intrinsic cisplatin-resistant TC cells (64). Furthermore, the high levels of cytoplasmic localized p21 in intrinsic cisplatin-resistant TC cells were essential to protect these cells for the cisplatin-induced pro-apoptotic effect of CDK2 (64). Therefore, we conclude that one key determinant of cisplatin-resistance in TC cells is high cytoplasmic expression of p21.

Regulation of p21 protein levels in TC. A recent study has provided novel insight in the mechanism causing the low p21 levels in cisplatin-sensitive versus the high p21 levels in cisplatin-resistant TC (64). Involvement of the miR-106b seed family in regulating p21 expression and cell cycle control has previously been demonstrated (88,89). Additionally, it has been described that miR-17-5p, miR-106a and miR-20a belonging to the miR-106b seed family are under transcriptional control of Oct4 in murine ES cells (90). Our study shows that in cisplatin-resistant in human TC cells, high expression of p21 is related to low expression of Oct4 and miR-106b seed family members, while transfection with pre-miRs led to p21 suppression and to a concomitant increase in cisplatin sensitivity (64). Suppression of Oct4 in a cisplatin-sensitive TC cell line Tera lowered the expression level of miR-106a, miR-17-5p and miR-20a resulting in enhanced p21-3'UTR dependent luciferase translation and robustly induced p21 protein expression (64). This was accompanied by a strong reduction in cisplatin induced apoptosis. Interestingly, in chemo-refractory TC patients almost no miR-17-5p, miR-106a and Oct4 staining was observed in EC components, with clearly detectable p21, while high levels of these miRs and Oct4 were detected in the EC component of chemo-sensitive TC patients.

Cytoplasmic p21, differentiation and cisplatin sensitivity in testicular cancer. Several studies have demonstrated that more differentiated TCs, such as teratomas, in contrast to EC, abundantly express p21 (58,59,61,62,91). Teratomas predominately show a nuclear localization of p21 and are neither growing, possibly via p21-mediated cell cycle arrest, nor responsive to treatment with cisplatin (3,8,63,91). After treatment with cisplatin-based che-

motherapy residual vital mature teratoma is found in 30-40 percent of the patients, implying a selection for more differentiated components that are less chemosensitive (63,92). However, occasionally an enlarging mature teratoma arises during or after chemotherapy (63,93), indicating a loss of cell cycle arrest. This is called the growing teratoma syndrome. These growing teratomas appeared to be responsive to a selective CDK4/6 inhibitor, PD0332991 (63). It is tempting to speculate about a possible redistribution of p21 from the nucleus to the cytoplasm in this syndrome, but so far no data are available. In contrast to teratomas, primary ECs hardly express any p21 (58,59,61,62). ECs are characterized by a capacity to differentiate, which is observed both in vitro and in vivo (94-98). Moreover, ECs share many similarities with embryonic stem cells (3,99) including their miRNA expression profile (99) and mRNA expression of a cassette of pluripotency genes, such as Oct4, Nanog and Stellar (3,99,100). In addition, retinoic acid (RA) treatment of the TC/EC cell line Tera resulted in repression of Oct4, Nanog and Stellar. Oct4 suppression has a similar effect as RA treatment, since RA treatment as well as Oct4 knockdown induced differentiation of Tera cells (100,101), and concomitantly induced resistance to cisplatin (102,103). Interestingly, after RA treatment or Oct4 suppression downregulation of miR-106 seed family members was observed (64,104). The miR-106b seed family is potentially oncogenic (105) and has been implied in embryonal stem cells maintenance, control of differentiation (106) and fine-tuning of p21 expression, via inhibition of p21 translation by binding to the p21'3UTR (64,88,89). Therefore, it is not surprising that the onset of differentiation in Tera cells leads to enhanced expression of p21 (107-109) that is mainly cytoplasmic localized 2 days after RA treatment or Oct4 suppression (64).

Together these observations show a direct correlation between cytoplasmic p21 expression, regulated by Oct4, miR-106b seed family members, and resistance of testicular cancers to cisplatin. In addition, these data strongly suggest a link between cytoplasmic p21 and onset of differentiation.

How to translate this knowledge to better therapeutic possibilities. P53 function is impeded by the interaction with MDM2 and the subsequent sequestration of p53 in the cytoplasm in cisplatin-resistant testicular cancer cells (Chapter 4). Therefore, targeting the p53/MDM2 axis may sensitize TC cells to cisplatin. Nutlin-3 inhibits the interaction between MDM2 and p53 by binding MDM2 in the p53-binding pocket leading to stabilization of p53 (29). Treatment with Nutlin-3 as a single agent induced apoptosis and at minimally cytotoxic concentrations nutlin-3 sensitized testicular cancer cells to cisplatin (Chapter 4 & refs 110,111). Both Nutlin-3 and siRNA against MDM2, caused a high induction of p53 and Fas membrane expression, a massive apoptosis induction, and a strongly reduced survival in both cisplatin-resistant and -sensitive testicular cancer cell lines (Chapter 4). Combining non-toxic concentrations of Nutlin-3 with cisplatin leads to a further activation of the p53 pathway and the Fas/FasL death receptor pathway and sensitizes both intrinsic as well as acquired cisplatin-resistant testicular cancer cells even to low concentrations of cisplatin (Chapter 4). Blocking of the Fas/FasL death receptor pathway impairs apoptosis induction of Nutlin-3 as well as Nutlin-3 combined with cisplatin in testicular cancer cells (Chapter 4). After Nutlin-3 treatment or cisplatin treatment, p21 mRNA and protein levels remained relatively low in testicular cancer cells (7,73,110). Though occupancy of p53 on the p21 promoter in testicular cancer cells showed similar levels compared to U2OS and HCT116 cells, a clear difference in p21 expression was observed between testicular cancer cells and non-testicular cancer

cells (110). Therefore, it is legitimate to assume that the release of negative feedback on p53 turns the balance in favor of transcribing pro-apoptotic genes, such as proteins involved in the Fas death receptor pathway. The results discussed herein suggest that the activation of the complex DNA damage-induced p53 pathway, with MDM2 as feedback mechanism fine-tuning the p53 response, seems to play a crucial role in balancing chemotherapy response of testicular cancers. Moreover, efficient activation of the p53-induced pro-apoptotic pathway by antagonizing MDM2 overrules the anti-apoptotic effect of p21 in response to cisplatin-induced DNA damage in testicular cancers.

Increased levels of cytoplasmic localized p21 is a key determinant of cisplatin-resistance in TC cells (64). Cytoplasmic localization of p21 is caused by PI3K/Akt mediated phosphorylation of p21 (64). Cytoplasmic localized p21 can be re-localized towards the nucleus by dephosphorylation of p21, using Triciribine, an Akt inhibitor currently in phase 1 trial, siRNA against Akt or the PI3K inhibitor LY294002. Relocalization of p21 caused a loss of complex formation of p21 with pro-apoptotic CDK2 and sensitized cisplatin-resistant TC cells for cisplatin-induced apoptosis (64). Therefore, a therapeutically interesting approach would be targeting Akt phosphorylation with the use of PI3K/Akt inhibitors (112). Whether PI3K/Akt inhibitors currently in clinical trial, such as Triciribine, SF1126, BKM120, PX-866, BEZ235 and GDC-0941 are effective, needs to be elucidated.

Conclusions

Testicular cancer is considered a paradigm for curable advanced malignancy and therefore, an informative model to investigate and understand the molecular determinants of chemotherapy sensitivity (and resistance) of solid tumors. Extensive knowledge about mechanisms involved in sensitivity and resistance of testicular cancer to DNA-damaging agents contributes to the design of new treatment strategies to overcome cisplatin resistance of refractory testicular cancers and of other human cancers. Based on our own studies and literature presented, we conclude that presence of wild-type p53, almost no p21 induction, and activation of the Fas apoptotic pathway is important for the exquisite sensitivity of TC cells to cisplatin. The reduced levels of p21 are caused by high expression of Oct4 and miR-106b seed family members. In cisplatin-resistant TC cells, resistance is caused by high levels of cytoplasmic p21 and tight regulation of p53 by MDM2. P21 is predominantly localized in the cytoplasm and does not induce cell cycle arrest, but antagonizes the pro-apoptotic function of proteins downstream of Fas, such as Ask1 and CDK2 in these cisplatin-resistant TC cells. To overcome cisplatin resistance of TC cells, two main strategies have been identified. Relocalization of cytoplasmic p21, using inhibitors of the PI3K/Akt pathway, resulted in a strong sensitization to cisplatin. Alternatively, antagonizing the MDM2-mediated negative feed-back of p53 with nutlin-3 also largely enhanced cisplatin sensitivity in TC cells.

Wild-type p53 is present in most tumors of TC patients, while recent data showed that high levels of cytoplasmic p21 and low levels of Oct4 and miR106-b family members were features of EC tumors of refractory TC patients. Therefore, enhanced efficacy in the treatment of refractory testicular cancers may be achieved by the combination of conventional chemotherapy with MDM2 antagonists or PI3K/Akt-inhibitors. This needs to be further studied in preclinical and clinical settings. Gaining those insights may direct novel therapeutic approaches in other p21-expressing solid tumors with wild-type p53 as well.

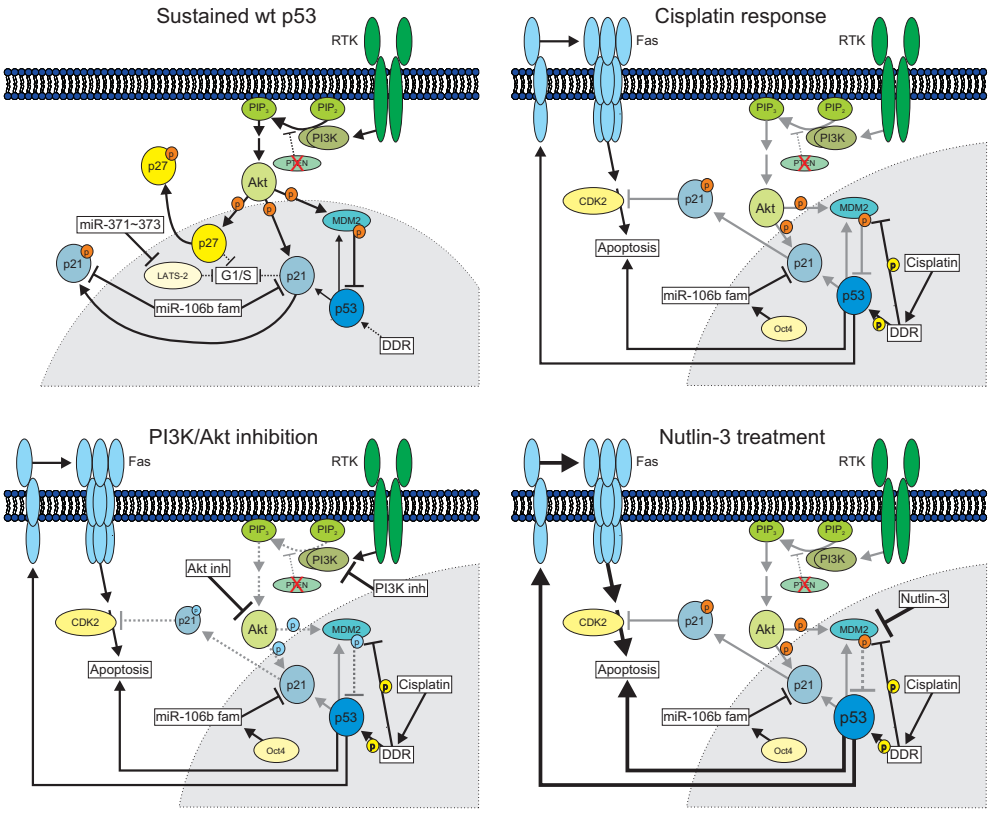

Figure 1. The role of wild-type p53, MDM2, p21 and Fas in cisplatin sensitivity and strategies to enhance cisplatin sensitivity in testicular cancer. (A) Schematic overview of connected pathways, which are causative for the sustained presence of wild-type p53 in testicular cancer. Solid lines represent active, while dotted lines represents inactive signaling. (B) Cisplatin-induced DNA damage activates p53, which in turn transcribes MDM2 and p21, and activates the Fas apoptosis pathway and the mitochondrial apoptosis pathway. MDM2 is important for regulating p53 trans-activational activity, p-Akt is important for dynamic shuttling of p21 from the nucleus towards the cytoplasm, while cytoplasmic p21 can block apoptosis. Oct4 regulated miR-106b seed family members (miR-106b fam) are involved in regulating p21 expression levels. Cisplatin-sensitive cells are characterized by high levels of Oct4 and miR-106b family members and as a consequence low amounts of cytoplasmic p21, resulting in cisplatin-induced apoptosis. Cisplatin-resistant cells have lower levels of Oct4 and miR-106b family members and high amounts of cytoplasmic p21, resulting in CDK2 inhibition and concomitantly moderate levels of cisplatin-induced apoptosis. Grey lines represent anti-apoptotic response and black lines represent pro-apoptotic response to cisplatin-induced DNA damage. (C) Deactivation of Akt with LY294002, Triciribine or siRNA against Akt sensitizes cisplatin-resistant cells. Deactivation of Akt leads to nuclear localization of p21, which in turn is no longer capable of blocking cisplatin-induced apoptosis. Dotted grey lines represent inactivated signaling, due to treatment. (D) Interfering in p53-MDM2 complex formation by Nutlin-3 treatment (or suppression of MDM2) substantially induced Fas expression, resulting in apoptosis of both cisplatin-sensitive and -resistant TC cells. Cisplatin in combination with Nutlin-3 further enhanced Fas expression and sensitized cisplatin-sensitive and -resistant TC cells to cisplatin-induced apoptosis. Thick black lines represent hyper-activated pro-apoptotic signaling, due to treatment.

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Chapter 6

Response to bleomycin in human testicular cancer cells is determined by bleomycin hydrolase levels

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Abstract

Bleomycin is an essential component of cisplatin-based chemotherapy regimens for patients with disseminated testicular cancer (TC), the paradigm of chemotherapy-sensitive solid tumors. However, a subset of these young patients still die because of chemo-resistant disease. Bleomycin hydrolase (BLMH) is an enzyme capable of metabolic inactivating of bleomycin. Increased enzymatic activity and elevated expression of BLMH have been observed in bleomycin-resistant human tumor cell lines. Besides, the homozygous variant G/G of the SNP A1450G in BLMH, thought to be involved in controlling the enzymatic activity, was associated with reduced survival and chemo-resistant disease in testicular cancer patients. In this study, we used a TC cell line panel, all homozygous wild-type A/A for the SNP A1450G, consisting of Tera, the acquired cisplatin-resistant subline Tera-CP, and the intrinsic cisplatin-resistant Scha cell line. Therefore, differences in enzymatic properties of BLMH due to different SNP genotypes of the cell lines used in the present study could be excluded. Highest BLMH levels were detected in the 10-fold bleomycin-resistant Scha cells. Tera and Tera-CP had similar BLMH levels indicating that acquired cisplatin-resistance is not related to bleomycin-resistance. High levels of BLMH coincided with a decreased sensitivity to bleomycin and a decrease in bleomcyin-induced apoptosis. Moreover, suppression of the high BLMH level using siRNA sensitized Scha cells to bleomycin-induced apoptosis but not to cisplatin. In conclusion, the level of BLMH is an important determinant of sensitivity to bleomycin in TC cells.

Introduction

Testicular cancer (TC) is the most common types of malignancy in men from 20-40 years of age, with a still increasing worldwide incidence (1). The mortality rate of TC has decreased, leading to a long-term survival of over 80% (2,3). This improvement in survival is related to the introduction of cisplatin for the treatment of disseminated TC in the late 1970s and the further development of cisplatin-based regimens (4-6). Bleomycin has proven to be an important and essential component of cisplatin-based chemotherapy regimens, like PVB (Cisplatin, Vinblastin, Bleomycin) and the currently used standard treatment regimen BEP (Bleomycin, Etoposide and Cisplatin), to treat patients with TC (7-9). In addition bleomycin is used in combination with other cytotoxic drug in the treatment of Hodgkin's disease.

However, the use of bleomycin as an anticancer drug is limited by bleomycin-induced pulmonary inflammation and fibrosis (pneumonitis) and tumor resistance to bleomycin. Pneumonitis is a bleomycin treatment related complication that occurs in approximately 10% of TC patients and is fatal in about 1% of the patients (10-13). Toxicity to bleomycin might be influenced by decreased drug clearance, repair of bleomycin-induced DNA lesions and/or inactivation of bleomycin (14-16). Bleomycin hydrolase (BLMH) is an enzyme capable of metabolically inactivating bleomycin (15) that shows low expression levels in human lungs and skin (17). In mice, lack of enzymatic activity of BLMH is associated with increased bleomycin-induced pulmonary toxicity (18,19). Moreover, increased enzymatic activity and elevated expression of BLMH in bleomycin-resistant human non-testicular tumor cell lines were observed (15,17,20,21), suggesting that activity and or levels of BLMH influence toxicity and antitumor efficacy of bleomycin. Recently we have defined that the homozygous variant G/G of the BLMH gene SNP A1450G is associated with reduced survival and higher chemo-resistant disease in patients with testicular cancer treated with cisplatin-bleomycin containing chemotherapy (22).

In the presented study we investigated the sensitivity of TC cell lines to bleomycin and cisplatin, and related the sensitivity to BLMH protein levels (and genotype). Moreover, we suppressed the cells for BLMH with siRNA to interfere with the cytotoxic response to bleomycin.

Materials and Methods

Chemicals. RPMI 1640, Optimem medium, HAM, F12-DMEM and Oligofectamine reagent were obtained from Invitrogen (Merelbeke, Belgium). Fetal calf serum (FCS) was purchased from Bodinco (Alkmaar, the Netherlands) and MTT (3-(4,5-dimethylthiazol-2-yl)-2,5diphenyl-tetrazoliumbromide), trypsin, and acridine orange from Sigma-Aldrich (Amsterdam, the Netherlands). Dimethyl sulfoxide (DMSO) was obtained from Merck (Darmstadt, Germany). Bleomycin and cisplatin were obtained from Pharmachemie (Haarlem, the Netherlands). Polyvinylidene difluoride (PVDF) membranes were obtained from Millipore BV, Etten-Leur, the Netherlands. The monoclonal antibody against BLMH was obtained from Abcam (Cambridge, UK) and the monoclonal antibody against beta-actin from (MP Biomedical, the Netherlands). The horseradisch peroxidase (HRP)-conjugated secondary rabbit anti-mouse antibody was from Dako (Glostrup, Denmark) and the Lumi-light PLUS western blot substrate kit from Roche Diagnostics (Almere, the Netherlands).

Cell Lines. The human germ cell tumor cell lines Tera, its cisplatin resistant sub-line Tera-CP, and the intrinsic cisplatin resistant Scha were used (23,24). All cell lines were cultured as monolayers in RPMI 1640 medium with 10% heat-inactivated FCS and harvested by scraping. Cells were cultured at 37°C in a humidified atmosphere containing 5% CO2. BLMH genotype, of the cell lines used in this study, was determined by polymerase chain reaction (PCR) and a restriction fragment length polymorphism technique, according to the previously described procedure (22).

Cytotoxicity. Sensitivity to bleomycin and cisplatin was tested with the microculture tetrazolium assay as described before (25). Cells were continuously incubated with the drugs in 96-wells tissue-culture plates in HAM/F12-DMEM medium (1:1) supplemented with 20% FCS. After a culture period of 4-days, 20 µl MTT solution (5 mg/ml) was added to each well for 225 min; then the plates were centrifuged, the supernatant was aspirated, and the formed formazan crystals were dissolved in 200 µl DMSO, after which the extinction was read at 520 nm using a Benchmark Microplate Reader (Biorad, Veenendaal, the Netherlands). The percentage of cell survival was calculated by dividing the mean of the test sample by the mean of the untreated samples. Controls consisted of media without cells (background extinction) and cells incubated with medium without drugs (growth control). At least three independent experiments were performed.

SDS-Polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting. Levels of the BLMH protein were determined by SDS-PAGE followed by western blotting. Cells were harvested as described above, washed once with cold phosphate buffered saline (PBS: 6.4 mM Na2HPO4, 1.5 mM KH2PO4, 0.14 M NaCl, 2.7 mM KCl, pH 7.2), lysed by standard Western blot sample buffer (50 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 5% 2-β-mercaptoethanol, 0.002% bromophenol blue) and boiled for 5 min to denaturize the proteins. Protein concentrations were determined according to Bradford(26). Total cell lysates were size-fractionated by electrophoresis (SDS-PAGE, Biorad, Veenendaal, the Netherlands) and transferred onto activated PVDF membranes using a tank-blot system (Biorad). Upon staining, the membranes were washed in Tris-buffered saline (TBS: 100 mM Tris-HCl, 137 mM NaCl, pH 7.8) and blocked for 1h in TBS supplemented with 5% milk powder and 0.05% Tween-20 (TBSt-milk). Immunodectection of BLMH was performed by incubation with the mouse anti-human BLMH (2079C4a 1:800 diluted in TBSt-milk) overnight at 4°C. Finally antibody binding was determined using HRP-conjugated secondary rabbit anti-mouse antibody and visualization was performed with the Lumi-light PLUS western blot substrate kit. Immunostaining of beta-actin (C4, 1:10000 diluted in TBSt-milk) was performed as a control for equal protein loading.

Apoptosis. Staining with acridine orange was used to distinguish apoptotic cells from vital cells (27). In the apoptosis experiments, 5,000-10,000 cells per well were pre-incubated with siRNA for 24h followed by incubation with bleomycin or cisplatin for 4h (short/high treatment) or 24 h (long/low treatment) in 96-wells micro-culture plates in a total volume of 200 µl. Untreated cells served as control. After 48 h of incubation at 37°C, acridine orange was added to a final concentration of 5 µg/ml and cells were incubated at 37°C for a further 10 min. Thereafter, 125 µl of the supernatant was carefully removed before proceeding with cell counting using a fluorescent microscope (Olympus IM, Japan) at 535 nm. The percentage of apoptotic cells was calculated by dividing the amount of apoptotic cells by the total amount of cells counting at least 300 cells per well.

Downregultation BLMH by RNA interference. Small interfering RNAs (siRNAs) were used for down-regulation of BLMH. Double-stranded BLMH siRNA molecules 5'-GCU CUG AUA CAG AAA CUG AAU dTdT-3' (sense) and 5'-AUU CAG UUU CUG UAU CAG AGC dTdT-3' (antisense) were designed and synthesized by Eurogentec (Maastricht, the Netherlands). Scrambled oligonucleotides, from Eurogentec (Maastricht, the Netherlands) served as negative control. Cells were plated in 6-wells or 96-wells plates in unsupplemented Optimem medium and transfected with 0.12 µM siRNA using Oligofectamine reagent according to the manusfacturer's protocol (Invitrogen) for respectively protein and apoptosis measurements.

Statistics. Statistical analysis consisted of a (un-)paired Student t-test. A double sided p-value < 0.05 was considered to indicate significance.

Results

High BLMH levels are associated with reduced bleomycin cytotoxicity and apoptosis-induction in TC cells. We used a TC cell line panel consisting of Tera, an acquired 3.1 fold cisplatin-resistant subline Tera-CP, and the intrinsic 13-fold cisplatin-resistant Scha cell line. Figure 1A shows the survival curve of Tera, Tera-CP and Scha after continuous incubation

with cisplatin for 96h with IC_{50} (concentration inhibiting survival by 50%) values indicated in Table 1. No difference in sensitivity to bleomycin after incubation for 96 h was observed between Tera and Tera-CP, whereas Scha showed to be 10-fold resistant to bleomycin compared to the Tera and Tera-CP (Figure 1B & Table 1).

Next, we determined BLMH expression levels in the TC cell lines by Western Blot analysis. Almost no difference in BLMH protein levels between Tera to Tera-CP was observed, while Scha expressed approximately 4-fold higher BLMH levels compared to Tera (Figure 1C). Cell lines were screened for a single nucleotide polymorphism (SNP) A1450G in the BLMH gene that is thought to be involved in controlling the enzymatic activity. All three TC cell lines showed to be wild type (A/A) for the SNP A1450G.

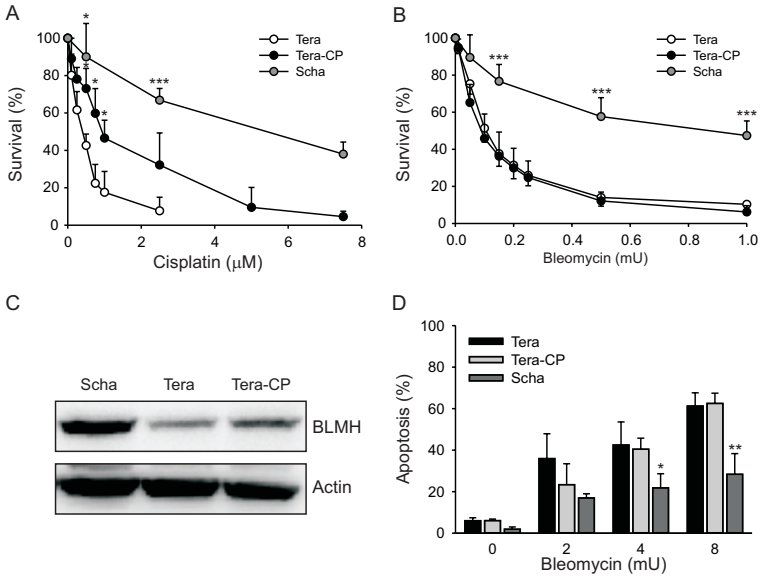

Figure 1. BLMH levels are associated with the response to bleomycin (A-B) Survival of TC cells after 96h of continuous cisplatin treatment (A) and bleomycin treatment (B) as indicated; values are the mean ± SD of three independent experiments; *p < 0.05; **p < 0.01; ***p < 0.005 Tera-CP or Scha compared to Tera for cisplatin and Scha compared to Tera/Tera-CP for bleomycin. (C) Western blot analysis showing the levels of BLMH of the TC cell lines Scha, Tera and Tera-CP. Actin was used as loading control; data are representative of three independent experiments. (D) Apoptotic response after 24h of treatment with bleomycin as indicated; values are the mean ± SD of three independent experiments; *p < 0.05; **p < 0.01; ***p < 0.005 Tera-CP or Scha compared to Tera.

We compared bleomycin-induced apoptosis in the TC cell lines. In Tera and Tera-CP, an almost similar concentration-dependent apoptotic response was observed after bleomycin treatment. In Scha, in contrast, bleomycin-induced apoptosis was strongly reduced compared to Tera and Tera-CP reflecting the results in the survival assay and indicated a relation with BLMH levels (Figure 1D).

High levels of BLMH protect TC cell line Scha for bleomycin-induced apoptosis. To investigate the importance of high levels of BLMH, TC cells were depleted of BLMH. Only treatment with specific siRNAs for BLMH showed a clear down-regulation of the target protein in all cell lines (Figure 2A, Tera-CP not shown). Down-regulation of BLMH led to

higher apoptosis levels in Scha cells upon bleomycin treatment compared to Scha cells transfected with scrambled siRNA (Figure 2B). In Tera cells, expressing low endogenous BLMH levels, no significant effect of BLMH suppression on bleomycin-induced apoptosis levels was observed (Figure 2B). No effect on cisplatin-induced apoptosis was observed in Tera and Scha cells suppressed for BLMH (data not shown).

The effect of BLMH levels on apoptosis was further investigated using a short incubation time (4h) with higher doses of bleomycin. In BLMH suppressed Scha cells bleomycin treatment even doubled the induction of apoptosis compared to control (Figure 2C). No effect of down-regulation of BLMH on cisplatin-induced apoptosis was observed in Scha cells using high doses and short treatment time (Figure 2D).

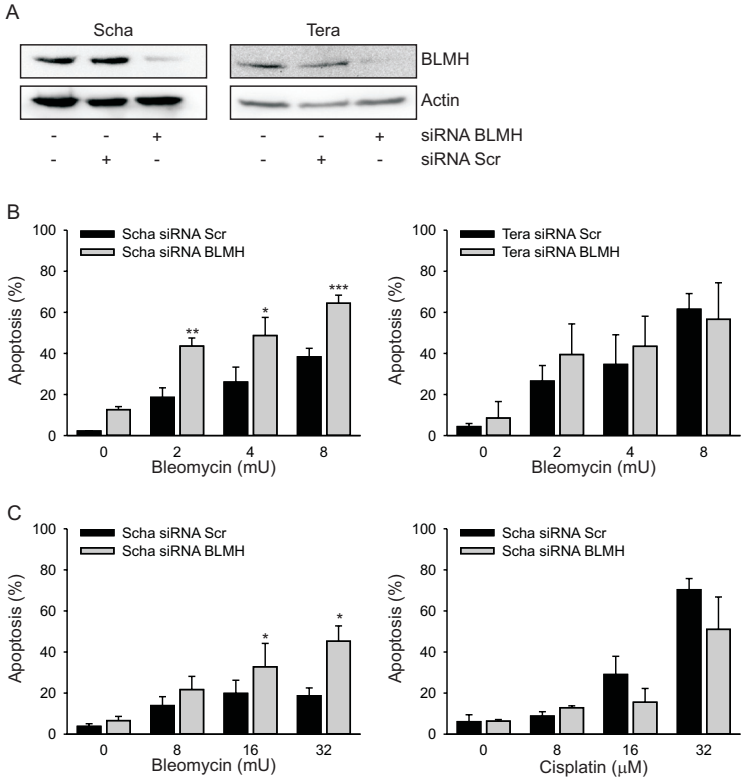

Figure 2. BLMH suppression sensitizes TC cells to bleomycin. (A) Successful downregulation of BLMH in the TC cells Tera and Scha. Data are representative of three independent experiments. (B) Suppression of BLMH increased the apoptotic response of the high BLMH expressing Scha cells 24h after continues bleomycin treatment as indicated, while no significant increase in the apoptotic response was observed in the low BLMH expressing Tera cells; values are the mean ± SD of three independent experiments; *p < 0.05; **p < 0.01; ***p < 0.005 Scha siRNA BLMH versus Scha siRNA Scr. (C) Suppression of BLMH increased the apoptotic response after a short incubation time (4h) and higher doses of bleomycin, as indicated, in Scha cells. While no significant increase in the apoptotic response was observed after short/high treatment with cisplatin in Scha cells; values are the mean ± SD of three independent experiments; *p < 0.05; **p < 0.01; ***p < 0.005 Scha siRNA BLMH versus Scha siRNA Scr.

Discussion

BLMH deaminates bleomycin and consequently converts bleomycin into the metabolite desamido-bleomycin, which in contrast to bleomycin poorly cleaves DNA and lacks cytotoxicity (18,28). Bleomycin is an essential component of the cisplatin-based chemotherapy regimens for treatment of TC. Since metabolic inactivation of bleomycin by BLMH appears to be associated with human tumor resistance to bleomycin (15), we investigated whether BLMH levels influence survival and apoptotic response in TC cell lines treated with bleomycin. Here, we show that high levels of BLMH are associated with a diminished apoptotic and cytotoxic response to bleomycin. No association between cisplatin sensitivity and BLMH levels was observed. Furthermore, suppression of BLMH sensitized the high BLMH expressing cells to bleomycin, but not to cisplatin.

Table 1 – IC_{50} values, resistant factor (RF), BLMH protein expression and BLMH genotype of the cell lines used in this study

	IC_{50} cisplatin (μM)[1]	IC_{50} bleomycin (mUSP)[2]	Rel. BLMH expression[3]	BLMH genotype
Tera	0.35 ± 0.06 (RF 1.0)	0.10 ± 0.02 (RF 1.0)	1	A/A
Tera-CP	1.07 ± 0.28 (RF 3.1)	0.09 ± 0.04 (RF 0.9)	1.25	A/A
Scha	4.81 ± 0.57 (RF 13)	0.95 ± 0.26 (RF 9.5)	4.01	A/A

1) The IC_{50} (drug concentration reducing cell survival by 50%) for cisplatin was calculated from the graph in Figure 1A. The mean IC_{50} ± SD was determined in three experiments, each performed in quadruplicate. Resistant factor (RF) was calculated by dividing the IC_{50} of the cell lines by the IC_{50} of Tera.

2) The IC_{50} for bleomycin was calculated from the graphs in Figure 1B. The mean IC_{50} ± SD was determined in three experiments, each performed in quadruplicate. RF was calculated by dividing the IC_{50} of the cell lines by the IC_{50} of Tera.

3) Relative BLMH expression (Rel. BLMH expression) was calculated with image J1.41 (National Institutes of Health) by dividing BLMH/Actin and normalized against Tera.

Other substrates of BLMH, besides bleomycin, homocysteine and probably amyloid precursor protein (29-31), are currently unknown. BLMH is supposed to have a conserved cellular function (17,19,32) and is suggested to play a role in protein processing and degradation, since it resembles a proteasome-like structure (33,34). Consequently, BLMH might influence degradation of proteins involved in the apoptotic response to cytotoxic drugs other than bleomycin, including cisplatin. Our results suggest that BLMH is not involved in regulating intracellular response pathways to cisplatin in TC.

The observed importance of BLMH levels in determining sensitivity to bleomycin is in agreement with previous studies on normal tissue and cancers discussed below. The sensitivity of lungs to bleomycin can best be explained by the lack of BLMH activity (18), due to low expression levels of BLMH observed in human lung tissue (17). High BLMH activity in lung tissue of rabbits actually protected the lungs against the toxic effects of bleomycin (18). High BLMH expression levels were observed in several bleomycin-resistant human tumor cell lines, including cell lines originating from Burkitt lymphomas, colorectal adenocarcinomas and leukemias (15,17,20,21). In contrast, low or undetectable levels of BLMH were observed in Hodgkin's disease (21), which is considered bleomycin-sensitive. Of interest, E-64, a cysteine proteinase specific inhibitor that inhibits BLMH activity, sensitized Daudi lymp-

homa and Colo-205 cells to bleomycin in vitro and in vivo (15,20). Whether pharmacological inhibition of BLMH is a therapeutic option for TC patients needs to be further investigated, since inhibition of BLMH might increase bleomycin-induced pulmonary toxicity as well.

Recently, we observed that the somatic homozygous variant G/G of BLMH SNP A1450G is associated with reduced survival and a higher prevalence of early relapses in TC patients treated with bleomycin-containing chemotherapy (22). It is plausible that this specific polymorphism in the BLMH gene might affect bleomycin metabolism and thus influence bleomycin toxicity and/or anti-tumor efficacy, because it is located in the C-terminal region that intrudes the active-site cleft of BLMH (32,35,36). Although the involved amino acid substitution would unlikely lead to change in protein conformation, the involved region forms a likely candidate for interaction with a protein regulating the positioning of the C-terminal arm (32) that intrudes the active-site cleft of BLMH and thereby influences substrate specificity as observed for a yeast BLMH homolog (32,35,36). In the present study, we used TC cell lines that were homozygous wild-type A/A for this SNP. Therefore, we can exclude SNP-dependent differences in enzymatic properties of BLMH in the cell lines used in the present study. Future studies on biomarkers for bleomycin efficacy in TC patients may not only focus on the presence of the G/G variant of the BLMH SNP A1450G but also on BLMH expression levels in testicular tumors.

In conclusion, our results suggest that BLMH is an important determinant of cellular metabolism of and consequently resistance to bleomycin in high BLMH expressing TC cells.

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Chapter 7

Summary & General Discussion

Summary

Testicular cancers (TC) represent the most frequent solid malignant tumor in men 20-40 years of age and the incidence of testicular cancer has been arising world-wide (1). Platinum-based chemotherapy has improved outcome of testicular cancer enormously. In other tumor types, for instance bladder, lung and ovarian cancer, responsiveness to platinum-based chemotherapy is often observed, but most of these patients eventually will relapse and die from platinum-resistant disease. Testicular cancers are cured at a rate of greater than 80% with conventional cisplatin-based chemotherapy even when highly advanced (1-2). Testicular cancer is, therefore, considered to be a paradigm of a platinum-sensitive solid tumor. However, the remaining 10~20% of patients are refractory to treatment and most will eventually die from progressive disease (2).

P53 is commonly found mutated in other tumor types (3-6), while high levels of wild-type p53 are expressed in testicular cancer (7-9). The p53 pathway is crucial for effective tumor suppression in humans and a major role for p53 in the response to chemotherapeutic drugs and the execution of apoptosis has been described (10-14). P53 plays a central role in the response to DNA-damage, induced by chemotherapeutic drugs such as cisplatin, and can activate both the extrinsic and intrinsic death signaling pathways (14-15). Together this suggests that p53 is an important factor in cisplatin hypersensitivity of TCs. Several in vivo and in vitro studies suggest an important role for p53 in TC cisplatin responses (16-21). In contrast, other studies have failed to support a role for p53 (22-23). To decipher the role and importance of p53 in testicular cancer this thesis focuses mainly on the functionality of wild-type p53 and activation of the p53-dependent apoptotic pathway in TC. Understanding why these tumors are curable with cisplatin-based chemotherapy could lead to the development of more effective treatment for refractory TCs and other solid tumors.

In vitro, cisplatin treatment of TC cells resulted in enhanced levels of p53 and murine double minute 2 (MDM2), activation of the Fas apoptotic pathway, and induction of apoptosis, while the expression levels of the cyclin-dependent kinase (CDK) inhibitor p21 were almost not affected in cisplatin-sensitive cells (8,24). In contrast, gamma-irradiation induced p53 and MDM2 levels and massive levels of cytoplasmic p21 without inducing apoptosis or cell cycle arrest in TC cells (8). These results suggested an important role for cytoplasmic localized p21 in preventing DNA damage-induced apoptosis in TC cells. Therefore we explored the role of cytoplasmic p21 in determining resistance to cisplatin of TC cells. In **Chapter 2**, we provided evidence that one key determinant of cisplatin-resistance in the embryonal carcinoma component of testicular cancer is high cytoplasmic expression of p21. The EC component of the majority of refractory testicular cancer patients exhibited high cytoplasmic p21 expression, which protected TC cell lines against cisplatin-induced apoptosis via CDK2 inhibition. Localization of p21 in the cytoplasm was critical for cisplatin-resistance since relocalization of p21 to the nucleus by Akt inhibition sensitized TC cell lines to cisplatin. We also demonstrated in TC cell lines and human tumor tissue that high cytoplasmic p21 expression and cisplatin-resistance of the embryonal carcinoma component of testicular cancer were inversely associated with the expression of Oct4 and miR-106b seed family members. Thus, targeting cytoplasmic p21, including by modulation of the Oct4/miR-106b/p21 pathway, may offer new strategies for the treatment of chemo-resistant testicular and other types of cancer.

In human TC, in contrast to murine TC, the role of p53 mutational status and functionality at basal and after chemotherapeutic drugs response remains elusive. Therefore, we analyzed in **Chapter 3** functionality of wild-type p53 in the unique cisplatin sensitivity of human TC using a short interfering (si)RNA approach. A panel of cisplatin sensitive TC cell lines (833KE and Tera), a subline with acquired cisplatin resistance (Tera-CP) and a panel of intrinsic resistant TC cell lines (Scha and 2102EP), all expressing wild-type p53 were used. Cisplatin sensitivity was related to apoptosis induction in the TC cell lines. P53 and p53-transcriptional targets MDM2 and p21 were expressed in all TC cell lines. Basal levels of MDM2 and p21 mRNA and protein were highest in Scha and 2102EP and depending on p53 transactivation. Following cisplatin exposure, expression levels of p53 increased within 6 h and further enhanced at 24 h with a subsequent increase in MDM2 mRNA and protein levels and Fas cell membrane levels in all TC cell lines. Previously, we demonstrated that high induction of p21 protein levels protected TC cell lines against Fas-mediated apoptosis following irradiation. In contrast, cisplatin treatment resulted in only a small induction of p21 mRNA and protein in these TC cells. Down-regulation of p53 with siRNA lowered cisplatin-induced apoptosis in Tera and Tera-CP, which was associated with a diminished Fas membrane expression. In contrast, p53 suppression augmented cisplatin-induced apoptosis in Scha and 2102EP and concomitantly strongly suppressed MDM2 and p21 mRNA and protein expression. Our results indicate that p53 is involved in transactivation of pro- and anti-apoptotic genes in TC cells. The opposite role of p53 in cisplatin-induced apoptosis demonstrates the importance of the cellular context on the p53 transactivation pathway among TC cell lines.

MDM2, as transcriptional target of p53, is the main negative feedback regulator of p53. By binding to the transactivation domain of p53, MDM2 is able to regulate p53 activity and stability via several mechanisms such as promoting p53 degradation through ubiquitination, stimulating p53 nuclear export, and inhibiting acetylation of p53 (25-27). Thus, p53 function might be impeded by the interaction with MDM2 and the subsequent sequestration of p53 in the cytoplasm in cisplatin-resistant TC cells. Therefore we sought a possible role for the negative feedback regulation of MDM2 on p53 in determining the response to cisplatin. In **Chapter 4**, we demonstrate that in intrinsic and acquired-cisplatin resistant TC cells p53 resides in a complex with (MDM2) after cisplatin treatment. Hyper-activation of the p53 pathway using the MDM2 antagonist Nutlin-3 in TC cells leads to nuclear localization of p53 and effective apoptosis induction as single agent. Targeting MDM2 with Nutlin-3 or short interfering RNA extremely sensitize resistant TC cells to cisplatin. The observed effects are dependent on the presence of wild-type p53, since mutant p53 expressing TC cells or wild-type p53 suppressed TC cells are resistant to Nutlin-3. These results indicate that targeting the p53/MDM2 axis, in combination with standard treatment, can be a powerful strategy to pursue in cisplatin-resistant or -refractory testicular cancers. Specifically, we show that the Fas death receptor pathway plays an important role in MDM2 antagonist-induced apoptosis in TC cells. Importantly, we have identified a similar mechanism in wt p53 expressing Hodgkin lymphoma and acute myeloid leukaemia cells, suggesting a common profile for Nutlin-3-induced apoptosis, which involves the Fas death receptor pathway.

In **Chapter 5** we discuss why testicular cancer is considered as paradigm of curable solid tumor and why it is an ideal model to investigate and understand the molecular determinants of chemotherapy sensitivity (and resistance) of solid tumors. This review summarizes the current knowledge on the biological basis of cisplatin-induced apoptosis and response to

chemotherapy in testicular cancer. Moreover, the fact that testicular cancer sustains high basal levels of wild-type p53 is discussed. In turn, presence of wild-type p53 is causative for the exquisite sensitivity to cisplatin of testicular cancers. Resistance to cisplatin occurs via changes in levels and balance of key-players, such as p21, Fas and MDM2, acting upon cisplatin-induced activation of the p53-pathway. Recent studies suggest that high levels of cytoplasmic localized p21 and tight regulation of p53 by MDM2 protect EC cells against cisplatin-induced apoptosis. Drugs targeting either the p53/MDM2 axis or cytoplasmic localized p21 showed, at least pre-clinically, a sensitizing effect to cisplatin treatment. These drugs may be further developed for the treatment of platinum resistant disease.

Apart from cisplatin, another important drug used as standard for testicular cancer treatment is bleomycin. In combination with cisplatin, bleomycin adds significantly to the improved outcome. Bleomycin hydrolase (BLMH) is an enzyme capable of metabolic inactivating of bleomycin. Recently a SNP in the BLMH gene was associated with outcome of testicular cancer. In **Chapter 6**, we used a TC cell line panel consisting of Tera, the acquired cisplatin-resistant subline Tera-CP, and the intrinsic cisplatin-resistant Scha cell line, all homozygous wild-type A/A for the SNP A1450G. Therefore, differences in enzymatic properties of BLMH due to different SNP genotypes of the cell lines used in the present study could be excluded. Highest BLMH levels were detected in the 10-fold bleomycin-resistant Scha cells. Tera and Tera-CP had similar BLMH levels indicating that acquired cisplatin-resistance is not related to bleomycin-resistance. High levels of BLMH coincided with a decreased sensitivity to bleomycin and a decrease in bleomcyin-induced apoptosis. Moreover, suppression of the high BLMH level using siRNA sensitized Scha cells to bleomycin-induced apoptosis but not to cisplatin. In conclusion the level of BLMH, especially when elevated, is an important determinant of sensitivity to bleomycin in TC cells.

General discussion and future perspectives

Activation of pro-survival pathways, such as the PI3K/Akt pathway, as well as inhibition of the apoptotic pathway, by *TP53* mutations for instance, can lead to chemotherapeutic drug resistance in tumors (28-29). Testicular cancers, however, express high levels of wild-type p53 and are most often extremely sensitive to cisplatin. In this thesis the importance of p53 and its downstream targets p21, Fas and MDM2 in determining the response to cisplatin in testicular cancer models is described. In the next section the p53 pathways and other pathways that might influence levels of p21, Fas and MDM2 are highlighted as subjects for further research to uncover new therapeutic targets to improve the response to (cisplatin based) chemotherapy.

Role of p21 as putative target in TC. High cytoplasmic expression of p21 is one key determinant of cisplatin-resistance in the embryonal carcinoma component of testicular cancer, and cytoplasmic localization of p21 is strongly dependent on PI3K/Akt activity (Chapter 2). High p-Akt levels might be the result of the lack of expression of PTEN (30-31) with or without the combined with activation of receptor tyrosine kinases (RTKs) in TCs. It is of therapeutic interest to identify which RTK(s) are important for Akt activity in TC, since inhibition of p-Akt retained p21 in the nucleus resulting in sensitization of high p21 expressing EC cells to cisplatin-induced apoptosis (Chapter 2). Specific targeting of these RTK(s) with antibodies or RTK inhibitors may be a therapeutic option, therefore mass spectrometry on enzymatically cleaved membrane peptides or FACS analysis should be performed to find

possible RTKs. Moreover, the efficacy of the various subclasses of PI3K- or Akt-inhibitors to inhibit Akt resulting in relocalization of p21 and subsequently enhance cisplatin sensitivity in refractory TC patients needs to be further elucidated.

Mature teratomas are extensively differentiated parts of testicular cancer that often reside after chemotherapy and that are non-dividing and relatively chemoresistant. In these mature teratomas, p-p21 is present, but still p21 is mainly nuclear localized (Chapter 2). Therefore, other factors involved in regulating cytoplasmic versus nuclear localization of p21 might as well play a role. Two interesting candidate proteins have been described. First, Brap2 that can function as a cytoplasmic retention protein for p21 (32). Second, BCCIP that is reported to cause nuclear localization of p21 independent of p21-NLS phosporylation (33). Moreover, BCCIP regulates p21 expression (34-35) and modulates CDK2 kinase activity via p21 (36). It would be of interest to study expression of BCCIP and Brap2 in TC and to compare levels in relation to p21 localization between the various histological subtypes such as teratoma and EC.

Levels of cytoplasmic p21 are regulated by expression of the miR-106b seed family, while the levels of the miR-106b seed family in turn are regulated by Oct4 (Chapter 2). All these factors are implied in maintenance and control of differentiation of embryonal stem cells (37-40). Loss of these factors is of consequence for drug sensitivity and susceptibility for apoptosis induction of EC (Chapter 2). Further research is needed to obtain more insight into the mechanism of differentiation of EC cells, using for instance an inducible promoter system to over-express p21 or pursue strategies to repress Oct4 and or miR-106b seed family members in Tera cells. While we observed less expression of Oct4 and members of the miR-106b seed family (Chapter 2), a recent study showed even a complete lack of Oct4 expression in cisplatin-resistant ECs (41). Thus, p21, Oct4 and miR-106b seed family might be biomarkers of bad prognosis. Of interest, miRNAs are stably detected in plasma en serum (42-43) and their expression patterns appear to be tissue-specific in human cancer (44). Further research is needed to determine if levels of the miR-106b seed family members in clinical samples can be used as a prognostic marker for testicular cancer and a predictive marker for outcome of TCs after standard treatment.

Role of the p53-MDM2 axis in TC. p53 has been shown to be important for apoptosis induction in testicular cancer cells upon DNA damage (Chapters 3-5 & refs 17-21,24,45,46-47), although the exact mechanism behind p53 mediated trans-activation of pro-apoptotic and anti-apoptotic genes determining apoptotic outcome in testicular cancer cells is still unclear. This is most evident in Chapter 3, were we observed a cell context dependent effect of p53 suppression on apoptosis induction. This effect might be due to differences in balance of Fas and p21 levels (Chapters 2-4). After cisplatin treatment, elevated Fas membrane expression levels and low p21 levels were detected in cisplatin-sensitive cells while higher p21 levels and only minor elevated Fas levels were found in cisplatin-resistant cells (Chapters 2-4 & ref 24). In contrast to cisplatin treatment, despite an almost a similar p53 increase, gamma-irradiation strongly induced p21 accumulation in the cytoplasm in both cisplatin-resistant Scha and cisplatin-sensitive Tera cells (Chapter 2 & ref 8). The observed induction of p21 was p53 dependent, since inactivation of p53 completely abolished irradiation-induced p21 upregulation in Tera cells (22). The differences between p21 and Fas levels might be explained by different regulation or expression of co-factors and negative feed-back regulators upon cisplatin versus gamma-irradiation, such as NF-Y, MDM2 and MDMX, regulating p53

transcriptional activity of pro- or anti-apoptotic genes.

NF-Y is a regulator of p53-mediated *FAS* transcription, while it represses *CDKN1A* promoter activity (48-49) leading to reduced p21 expression. NF-Y mediated transactivation is in a dose-dependent manner suppressed by MDM2 (50). In turn, by binding to the transactivation domain of p53, MDM2 is able to regulate p53 activity and stability via several mechanisms, such as promoting p53 degradation through ubiquitination, stimulating p53 nuclear export, and inhibiting acetylation of p53 (25-27). MDM2 and the related protein MDMX are p53 regulating proteins, suggested to be of great importance in tailoring p53 response (51-55). MDMX knockdown in TC cells resulted in higher p21 expression (56). Interestingly, either MDM2 suppression or interference with the p53-MDM2 interaction using Nutlin-3 led to a robust upregulation of Fas membrane expression (Chapter 4), while after both Nutlin-3 and cisplatin treatment p21 mRNA and protein levels were very low in testicular cancer cells (8,24,56-57). Further research is needed to gain more insight in how the diverse co-factors/regulators affect p53-mediated pro- and anti-apoptotic response in TC upon different DNA damage inducing treatments. The application of p53 CHIP analysis and mass-spec on immuno-precipitated p53 complexes might be pursued to find differential p53 co-factor complexes.

We show that in cisplatin-resistant TC cells p53 function is impeded by the interaction with MDM2 and the subsequent sequestration of p53 in the cytoplasm (Chapter 4). More attention should be given to the role of two additional factors affecting the p53-MDM2 axis, in particular MDM2 phosphorylation, in TC, which are the ATM/ATR family protein kinases and the PI3K/Akt pathway. In general, DNA damage activates the ATM/ATR family protein-kinases and ATM/ATR fine-tunes the response of p53 by regulating additional post-translational modifications leading to stabilization of the normally labile p53 protein (58) and by affecting the p53 regulators MDM2 and MDMX. ATM directly and indirectly induces MDM2 and MDMX phosphorylation, resulting in decreased activity and stability (59-60) and consequently loss of negative feedback on p53. In contrast, phosporylation of MDM2 by p-Akt leads to the nuclear localization of MDM2 and consequently enhances MDM2-mediated ubiquitination, sequestration and degradation of p53 (61-62). The balance between Akt versus ATM/ATR induced phosporylation of MDM2 might thus play an important role in determining the response to cisplatin, but this was not assessed in TC cells.

Induction of FasL and upregulation of the Fas receptor in a p53-dependent manner has been observed in several tumor cell lines after treatment with chemotherapeutic drugs, such as cisplatin, and is related to apoptosis induction (8,24,46-47,63-65). The Fas/FasL system is active and functional in cisplatin-sensitive but almost inactive in cisplatin-resistant TC cells (24). Interestingly, the massive apoptosis induction in both cisplatin-sensitive and -resistant TC cells after Nutlin-3 treatment or Nutlin-3 in combination with cisplatin is to a large extent dependent on activation of the Fas death receptor pathway. This can be contributed to the strongly enhanced Fas membrane expression levels in TC cells (Chapter 4). Levels of FasL were also determined, by western blotting but no FasL upregulation could be detected, while suppression and blocking of FasL significant reduced the apoptotic response. This is however not conclusive to define the importance of FasL levels or distribution of FasL for efficient apoptosis induction, as this technique cannot distinguish between cellular, soluble or membrane-bound FasL. Therefore, further research using for instance different constructs of FasL resulting in either membrane-bound or soluble FasL should be performed to gain insight in the importance of FasL redistribution to the membrane or to the extracel-

lular compartment, i.e. membrane-bound vs. soluble FasL, in inducing apoptosis. Moreover, involvement of other genes in this pathway may be of interest. The Fas adaptor *LRDD* and a gene implicated in positive Fas regulation, *PHLDA3* were found to be regulated by p53 upon cisplatin treatment in Tera (20), but the effect of Nutlin-3 treatment on their expression and activation remains unknown up to now. Therefore, microarray analysis could be performed to gain more insight in Nutlin-3 induced gene expression in cisplatin-resistant vs. -sensitive TC cells in the presence and absence of cisplatin or Nutlin-3.

Resistance to bleomycin in TC. In chapter 6, we concluded that the level of BLMH is an important determinant of sensitivity to bleomycin in TC cells. Recently, the somatic homozygous variant G/G for the single-nucleotide polymorphism (SNP) A1450G in BLMH was reported to be associated with reduced survival and a higher prevalence of early relapses in TC patients treated with bleomycin-containing chemotherapy (66). It is plausible that this specific polymorphism in the BLMH gene might affect bleomycin metabolism and thus influence bleomycin toxicity and/or anti-tumor efficacy. For that reason, future research has to determine if this SNP in the BLMH gene, by more stable expression or increased activity of BLMH, may influence enzymatic activity and thereby the response to bleomycin.

Conclusions

Testicular cancer is considered a paradigm of curable solid tumor and is therefore a very helpful "model" to investigate and understand the molecular determinants of chemotherapy sensitivity (and resistance) of solid tumors. Knowledge about mechanisms involved in the sensitivity and resistance of testicular cancer to DNA-damaging agents might contribute to the design of new treatment strategies to overcome cisplatin resistance of refractory testicular cancers and of other types of human cancers. Based on literature and the studies presented in this thesis, we show data suggesting that presence of wild-type p53 is causative for the cisplatin sensitivity in testicular cancer (chapter 3, 4 and 5), while resistance to cisplatin occurs via changes in levels of cytoplasmic p21 (chapter 2) and tight regulation of p53 by MDM2 (chapter 4) in cisplatin-resistant cells. Additionally, expression levels of BLMH might determine the response to bleomycin (chapter 6), an important and essential component of the cisplatin-based chemotherapy regimens used in treatment of testicular cancers. In pre-clinical studies enhanced cisplatin efficacy after modulation of p21, p53 and MDM2 is shown. Translation of these results to the clinic can lead to new treatments of (refractory) testicular cancers by the combination of conventional chemotherapy with the application of MDM2 antagonist or PI3K- or Akt-inhibitors, in order to induce pro-apoptotic proteins or to impair the localization of anti-apoptotic proteins.

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Chapter 8

Nederlandse samenvatting

Introductie

Zaadbalkanker (testiskanker of kiemceltumor van de testis) is de meest voorkomende vorm van kanker bij mannen tussen de 20 en 35 jaar. De frequentie waarmee zaadbalkanker zich voordoet neemt wereldwijd toe (1). Op basis van histologie kunnen deze tumoren worden onderverdeeld in seminomateuze en non-seminomateuze zaadbalkanker. Non-seminomen hebben de neiging om op grote schaal te metastaseren (uitzaaien) en zijn daarnaast minder gevoelig voor radiotherapie dan seminomateuze zaadbalkanker. Dit heeft consequenties voor de prognose en voor de behandelingsstrategieën. Non-seminomen laten verschillende stadia van differentiatie zien, variërend van het ongedifferentieerde embryonale carcinoom (EC) tot de meer gedifferentieerde componenten zoals extra-embryonale dooierzak carcinoom (Yolk Sac YS) en choriocarcinoom (Chc) tot zeer gedifferentieerde teratoom (T) (2).

Op platina gebaseerde chemotherapie is de hoeksteen van de behandeling van vele verschillende maligniteiten en wordt gebruikt als eerstelijns therapie voor zaadbal-, blaas-, long- en eierstok-kanker. In het geval van blaas-, long- en eierstokkanker is de eerste reactie op platina gebaseerde chemotherapie zeer goed, echter bij de meerderheid van deze patiënten is de respons van beperkte duur. De meeste patiënten zullen uiteindelijk recidiveren met een platina resistente, refractaire tumor. Zaadbalkanker wordt beschouwd als een paradigma van een platina gevoelige solide tumor. Sinds de invoering van cisplatine in het midden van de jaren 1970 is er een enorme verbetering opgetreden in de effectiviteit van de behandeling en overlevingskansen voor patiënten met zaadbalkanker, zelfs als er sprake is van uitgebreid gemetastaseerde ziekte (3). Bouwend op het aanvankelijke succes, is de behandeling in de loop van jaren steeds verder verbeterd (4-5). Dit heeft geleid tot de huidige standaard behandeling voor zaadbalkanker, de combinatie chemotherapie BEP waarin bleomycine naast cisplatine en etoposide is opgenomen als een essentieel onderdeel. (6)

Patiënten met gemetastaseerde zaadbalkanker kunnen worden ingedeeld volgens de International Germ Cell Consensus Classification (internationale kiemcel consensus classificatie), deze onderscheid zaadbalkanker patiënten op de hoeveelheid aanwezige tumor. Drie groepen worden onderscheiden, namelijk patiënten met een goede -, een intermediaire - of een slechte prognose. De behandeling met op cisplatine gebaseerde chemotherapie combinatie zorgt voor een 5-jaars overleving van respectievelijk 91%, 79% en 48% in de hiervoor beschreven groepen (7). Ondanks het feit dat de totale behandeling meestal succesvol is zal ongeveer 20 ~ 50% van de zaadbalkanker patiënten met uitgebreid metastasering, behorende tot de intermediaire of slechte risico groep, geen langdurige remissie tonen na de eerste behandeling en uiteindelijk sterven als gevolg van hun ziekte (5).

Cisplatine moet worden geactiveerd door intracellulair hydratatie van een of beide van de twee chloride liganden voordat het covalent kan binden aan DNA (8). Terwijl bleomycine DNA klievende activiteit heeft wanneer het in complex is met een zuurstofhoudend ijzer molecuul (9). Na de behandeling met cisplatine worden letale DNA adducten gevormd (8-9). Wat zorgt voor een arrest in de DNA replicatie vork en verstoringen in het DNA die worden herkend door complexen van DNA-schade herkennende cellulaire eiwitten (8,10). Deze activeren op hun beurt verschillende signaaltransductie routes, betrokken bij het herkennen van DNA schade, met als gevolg celcyclus arrest (remming van de celdeling) en reparatie van DNA schade óf apoptose (geprogrammeerde celdood) (8,10-11). Resistentie tegen cisplatine of bleomycine kan het gevolg zijn van verminderde opname -, toegenomen export -, intracellulaire detoxificatie (afbraak) van de chemotherapeutica, een verhoogde efficiëntie van DNA reparerende systemen (8,10-13) en defecten in de apoptose route (14).

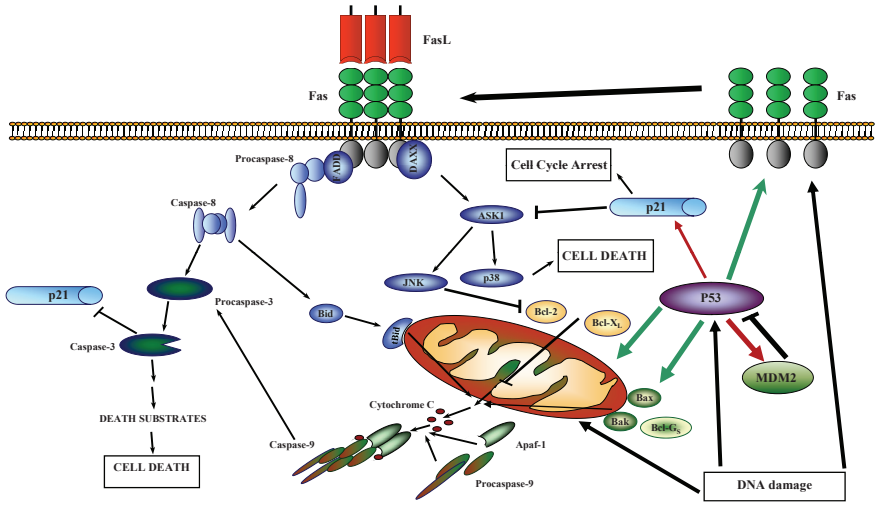

Figure 1. Apoptose of geprogrammeerde celdood is een actief proces in de cel en wordt gekenmerkt door biochemische en morfologische veranderingen. Het p53 tumor suppressor eiwit speelt hierin een centrale rol. De reactie op de door cisplatine chemotherapie veroorzaakte DNA schade kan zowel de extrinsieke apoptose route (met inbegrip van Fas / FasL) als de intrinsieke (mitochondriale) apoptose route, bijvoorbeeld Bax en Bak activeren. Dit leidt tot verhoogde expressie van pro-apototische genen (groene lijnen) en anti-apoptotische genen (rode lijnen). Deze activeren verschillende signaaltransductie routes die betrokken zijn bij DNA schade herkenning waarop cel cyclus arrest en reparatie (via p21) of apoptose volgen. Bij ernstige schade aan het DNA zal door pro-apoptotische eiwitten een proteolytisch systeem van caspases, een familie van proteasen geactiveerd worden. Deze leiden uiteindelijk tot het knippen van substraat eiwitten, resulterend in de totale demontage van de cel. Verdere uitleg is te vinden in de tekst. De figuur is een bewerking van eerdere figuren uit Di Pietro et al, 2005 en Spierings et al, 2003; referenties (22-23).

Apoptose, of geprogrammeerde celdood, is een actief proces in de cel dat wordt gekenmerkt door biochemische en morfologische (vorm) veranderingen (Figuur 1). Apoptose is betrokken bij de ontwikkeling en homeostase van normale weefsels (15), waaronder ook normaal testis weefsel. Tijdens de spermatogenese treedt apoptose op in de testis, als een belangrijk fysiologisch mechanisme voor kwaliteitsbewaking en aanpassing van de hoeveelheid kiemcellen aan de hoeveelheid ondersteunende Sertoli cellen (16-17). Een centraal onderdeel van het apoptose proces is de proteolyse uitgevoerd door een familie van proteasen, de caspasen. Het knippen van een set van substraateiwitten (proteolyse), door de caspasen, leidt uiteindelijk tot de totale demontage van de cel (Figuur 1). Dit proces kan worden aangestuurd door twee belangrijke apoptose signaalroutes namelijk de “extrinsieke”, death (dood) receptor, apoptose route en de “intrinsieke”, mitochondriële, apoptose route (15) (Figuur 1). Zowel de extrinsieke als intrinsieke signaalroute kan geactiveerd worden door het p53 tumor suppressor eiwit, dat een centrale rol speelt in de reactie op DNA schade, veroorzaakt door chemotherapeutische middelen (14) (Figuur 1).

P53 is een tumorsuppressor eiwit met een dubbele rol in de reactie op stress (zoals na chemotherapie). Het kan enerzijds genen transactiveren die apoptose induceren (zoals de Fas death receptor) en anderzijds genen transactiveren die aanzetten tot celcyclus arrest, zoals CDKN1A (coderend voor p21cip1/waf1). Zelf wordt p53 via een negatieve feedback geregu-

leerd door MDM2, deze remt de transactivatie activiteit van p53 en reguleert de afbraak van p53 (18) (Figuur 1). P53 is het meest frequent gemuteerde gen in menselijke maligniteiten (19-20), maar is verrassend genoeg zelden tot nooit gemuteerd in zaadbalkanker (21). Bij de patiënten leiden mutaties in p53 tot cisplatine resistentie door de beïnvloeding van de apoptotische route (21). De aanwezigheid van wild-type p53 zou daarom een verklaring kunnen zijn voor de gevoeligheid voor cisplatine (22-23), vooral omdat er ook véél p53 tot expressie komt bij de meeste kiemceltumoren (24-27). Er zijn echter ook onderzoeken beschreven waarin geen rol voor wild-type p53 in de gevoeligheid voor chemotherapie werd gevonden (28-30). In recent onderzoek is aangetoond dat de blootstelling van zaadbalkankercellijnen aan cisplatine resulteerde in verhoogde niveaus van p53 en MDM2, activatie van de Fas apoptotische route, en inductie van apoptose, zonder verandering van de expressie niveaus van p21 (25,31). Deze resultaten tonen de betrokkenheid aan van de Fas apoptotische route bij cisplatine geïnduceerde apoptose in zaadbalkanker cellen (25,31). Het is echter niet bekend waarom cisplatine geïnduceerde activatie van p53 niet resulteerde in de verhoogde expressie van p21. Dit suggereert dat veranderingen in de balans van bepaalde sleuteleiwitten in de cisplatine geïnduceerde, p53-gemedieerde, apoptose route het lot bepalen van zaadbalkankercellen na behandeling met cisplatine. Het ontcijferen van de complexe regulatie van deze moleculaire determinanten van cisplatine gevoeligheid en resistentie in de behandeling van zaadbalkanker, kan leiden tot betere behandelingsmogelijkheden voor patiënten met een resistent vorm van zaadbalkanker en andere solide tumoren.

Doelstelling van dit proefschrift

Hoewel p53 één van de meest bestudeerde eiwitten is in kankercellen, is de precieze rol van wild-type p53 in de response op DNA schade in zaadbalkanker niet duidelijk. Om deze reden richt dit proefschrift zich voornamelijk op de functie van wild-type p53 en de door p53 geïnduceerde eiwitten Fas, MDM2 en p21 en hun rol in cisplatine geïnduceerde apoptose in zaadbalkanker.

Samenvatting van de resultaten

Behandeling van zaadbalkanker cellijnen met cisplatine resulteerde in verhoogde expressie van p53 en MDM2, activering van de Fas apoptotische route én inductie van apoptose, terwijl in cisplatine gevoelige zaadbalkanker cellen de expressie van de cycline afhankelijke kinase (CDK) remmer p21 bijna niet steeg (25,31). Behandeling van zaadbalkanker cellijnen met gamma-straling daarentegen leidde tot verhoogde expressie van p53, MDM2 én cytoplasmatisch p21, zonder inductie van apoptose of celcyclus arrest (25). Deze resultaten suggereren een belangrijke rol voor cytoplasmatisch gelokaliseerd p21 in het voorkómen van apoptose na DNA schade in zaadbalkanker cellen. Daarom onderzochten wij de rol van cytoplasmatisch p21 in cisplatine resistentie van zaadbalkanker cellen. In **Hoofdstuk 2** laten we zien dat een hoge concentratie van cytoplasmatisch gelokaliseerd p21 een doorslaggevende factor is voor de cisplatine resistentie in de embryonale carcinoom (EC) component van zaadbalkanker. De EC-component van de meerderheid van de patiënten met refractaire zaadbalkanker bracht cytoplasmatische p21 hoog tot expressie. Daarnaast vonden wij dat in cisplatine resistente cellijnen p21 bescherming bood tegen cisplatine geïnduceerde apoptose via CDK2 remming. Lokalisatie van p21 in het cytoplasma was van cruciaal belang voor de resistentie tegen cisplatine, omdat realloceren van p21 naar de kern, door Akt remming, zaadbalkanker cellijnen gevoeliger maakte voor cisplatine. Ook hebben we in zaadbalkanker cellijnen en

in tumorweefsel van patiënten aangetoond, dat hoge expressie van cytoplasmatische p21 en resistentie tegen cisplatine in de embryonale carcinoom component van zaadbalkanker, omgekeerd evenredig is met de expressie van Oct4 en mir-106b familieleden. De modulatie van de Oct4/miR-106b/p21 route zou, via de verlaging van cytoplasmatisch p21, een aanknopingspunt kunnen zijn voor het ontwikkelen van nieuwe strategieën voor de behandeling van cisplatine resistente zaadbalkanker en andere vormen van kanker.

In humaan zaadbalkanker, is de rol van p53 mutatie status en het effect hiervan op de basale p53 functionaliteit en de functionaliteit na chemotherapie nog niet uitgekristalliseerd. Opmerkelijk hierbij is dat er een duidelijk verschil lijkt te zijn met muismodellen. Daarom hebben we in **Hoofdstuk 3** de functionaliteit van wild-type p53 geanalyseerd met betrekking tot de unieke cisplatine gevoeligheid van de humane zaadbalkanker cellijnen met behulp van "short interfering" (si)RNA, specifieke korte stukjes RNA die de aanmaak van één eiwit remmen. Voor dit onderzoek werden cisplatine gevoelige zaadbalkanker cellijnen (833KE en Tera), een cellijn met verworven cisplatine ongevoeligheid (Tera-CP) en intrinsiek cisplatine resistente zaadbalkanker cellijnen (Scha en 2102EP), alle met wild-type p53 expressie, gebruikt. De gevoeligheid voor cisplatine was gerelateerd aan de inductie van apoptose in alle cellijnen. P53 en de p53-transcriptionele geïnduceerde eiwitten, MDM2 en p21, kwamen in alle zaadbalkanker cellijnen tot expressie. Basale niveaus van MDM2 en p21, zowel van mRNA als eiwit, waren het hoogst in Scha en 2102EP en afhankelijk van transactivatie door p53. Blootstelling aan cisplatine induceerde al binnen 6 uur p53 en de expressie nam verder toe na 24 uur, gevolgd door inductie van MDM2, zowel op mRNA als eiwit niveau, en sterk verhoogde expressie van Fas op de celmembraan in alle zaadbalkanker cellijnen. Eerder hebben we aangetoond dat, na bestraling, een sterke inductie van p21 zaadbalkanker cellijnen beschermt tegen Fas-gemediëerde apoptose. In tegenstelling daarmee resulteerde behandeling met cisplatine in slechts een lichte inductie van p21 mRNA en p21 eiwit in dezelfde cellijnen. Remming van p53 expressie met siRNA verlaagde de cisplatine geïnduceerde apoptose in Tera en Tera-CP en was geassocieerd met een verminderde expressie van Fas op de celmembraan. Remming van p53 in Scha en 2102EP leidde tot een verhoogde cisplatine geïnduceerde apoptose, waarbij de expressie van MDM2 en p21, mRNA en eiwit sterk onderdrukt werd. Onze resultaten geven aan dat p53 betrokken is bij transactivatie van zowel pro- als anti-apoptotische genen in zaadbalkanker cellijnen. Het tegenovergestelde effect van remming van p53 op cisplatine geïnduceerde apoptose in verschillende zaadbalkanker cellijnen demonstreert het belang van de cellulaire context voor de rol van de p53 transactivatie route.

MDM2, een door p53 transcriptioneel geïnduceerd eiwit, is de belangrijkste regulator van de negatieve feedback op p53. Door binding aan het transactivatie domein van p53 is MDM2 in staat om, via verschillende mechanismen, de stabiliteit en de activiteit van p53 te reguleren. MDM2 bevordert ubiquitinatie van p53, en kan de nucleaire export en acetylatie van p53 stimuleren (32-34). In cisplatine resistente zaadbalkanker cellen zou het zo kunnen zijn dat de p53 functie wordt belemmerd door de interactie met MDM2 met verschuiving van p53 naar het cytoplasma als gevolg. Daarom hebben we de mogelijke rol voor de negatieve feedback regulatie van MDM2 op p53 in de reactie op cisplatine onderzocht in **Hoofdstuk 4**. Na blootstelling van zaadbalkanker cellijnen met intrinsieke en verworven cisplatine resistentie aan cisplatine is p53 ondanks de sterke toename in expressie nog steeds in complex is met MDM2. Hyper-activatie van de p53 route met behulp van de MDM2-antagonist Nutlin-3 brengt p53 terug naar de kern van de cel met effectieve inductie van apoptose als gevolg. Het

remmen van MDM2 activiteit met Nutlin-3 of remmen van MDM2 expressie met siRNA maakte de cisplatine resistente zaadbalkanker cellijnen gevoeliger voor cisplatine. De waargenomen effecten zijn afhankelijk van de toename van wild-type p53 activiteit, want zaadbalkanker cellen met mutant p53 of zaadbalkanker cellen waarin de expressie van wild-type p53 onderdrukt is, zijn ongevoelig voor Nutlin-3. Daarbij blijkt de Fas death receptor route een belangrijk mechanisme in het voltrekken van apoptose na blootstelling aan deze MDM2 antagonist, nutlin-3. Deze resultaten suggereren dat gerichte remming van de p53/MDM2 interactie, in combinatie met de standaard chemotherapie behandeling, een krachtige strategie is, die in de behandeling van cisplatine resistente zaadbalkankers zeker verder onderzocht en overwogen moet worden. Dat dit mechanisme zich niet beperkt tot zaadbalkanker cellijnen blijkt uit het feit dat ook in een Hodgkin cellijnen en acute myeloïde leukemie cellijnen met wild type p53, een sterke inductie van apoptose via de Fas death receptoren werd gevonden na behandeling met Nutlin-3.

In **Hoofdstuk 5** bespreken we waarom zaadbalkanker wordt beschouwd als paradigma van een te genezen vorm van kanker en tevens waarom het een ideaal "model" is om meer inzicht te krijgen in de moleculaire determinanten die bepalend zijn voor de chemotherapie gevoeligheid (en resistentie) van solide tumoren. In dit literatuur overzicht wordt actuele kennis van de biologische basis van cisplatine geïnduceerde apoptose en de respons op chemotherapie van zaadbalkanker belicht. In dit kader werd nader gekeken naar het feit dat zaadbalkankers basaal een hoge expressie hebben van wild-type p53 en hoe de aanwezigheid van wild-type p53 bepalend is voor de uitzonderlijke gevoeligheid voor cisplatine. Ongevoeligheid voor cisplatine kan verklaard worden door veranderingen in expressie en balans van door p53 transcriptioneel geïnduceerde sleuteleiwitten in de apoptose route zoals p21, Fas en MDM2. Recente studies laten zien dat hoge expressie van cytoplasmatisch gelokaliseerd p21 en of stringente regulering van p53, door MDM2, EC cellen beschermt tegen cisplatine geïnduceerde apoptose. Medicatie die zich richt op de p53/MDM2 as of op cytoplasmatisch gelokaliseerd p21 heeft, ten minste preklinisch, een sensibiliserend effect op de behandeling met cisplatine. Deze medicijnen kunnen verder ontwikkeld worden voor de behandeling van cisplatine resistente (zaadbal)kanker.

Naast cisplatine is bleomycine een belangrijk component van het chemotherapieschema dat standaard gebruikt wordt voor de behandeling van zaadbalkanker. In combinatie met cisplatine, draagt bleomycine in belangrijke mate bij aan het goede klinische resultaat. Bleomycine hydrolase (BLMH) is een enzym dat in staat is om bleomycine metabool te inactiveren. Onlangs werd een SNP in het BLMH gen geassocieerd met behandeluitkomsten van zaadbalkanker. In **Hoofdstuk 6**, gebruikten we een panel van zaadbalkankercellijnen bestaande uit; Tera, Tera-CP, een Tera-sublijn met verworven cisplatine resistentie, en Scha, een intrinsieke cisplatine resistente cellijn. Alle drie de cellijnen zijn homozygoot wild-type A/A, voor de SNP A1450G van het BLMH. Daarom kunnen verschillen in enzymatische eigenschappen van BLMH, als gevolg van de verschillende SNP genotypen van de in deze studie gebruikte cellijnen worden uitgesloten. BLMH komt het hoogst tot expressie in de 10-voudige bleomycine resistente cellijn Scha. Tera en Tera-CP hadden vergelijkbare BLMH expressie niveaus, waaruit blijkt dat verworven cisplatine resistentie niet verbonden is met bleomycine resistentie. De hoge BLMH expressie viel samen met een verminderde gevoeligheid voor bleomycine en een afname in bleomcyin geïnduceerde apoptose. Remming van de BLMH expressie, met behulp van siRNA, maakte Scha cellen gevoeliger voor bleomycine geïnduceerde apoptose, maar niet voor cisplatine geïnduceerde apoptose. Hoge

expressie van BLMH is een belangrijke determinant van óngevoeligheid voor bleomycine in zaadbalkanker cellen.

Conclusies

Zaadbalkanker wordt beschouwd als een paradigma voor een te genezen vorm van solide tumoren. Dit maakt het een zeer behulpzaam "model" voor onderzoek naar moleculaire determinanten die bepalend zijn voor de chemotherapie gevoeligheid (en resistentie) van solide tumoren. Kennis over de mechanismen die betrokken zijn bij de gevoeligheid en de resistentie van zaadbalkanker voor DNA schade inducerende medicijnen, kan bijdragen aan de ontwikkeling van nieuwe behandelstrategieën om cisplatine resistentie te overwinnen in refractaire zaadbalkanker en andere vormen van humane kanker. Gebaseerd op de in dit proefschrift beschreven literatuur en studies, tonen we aan dat de aanwezigheid van wild-type p53 in zaadbalkanker bepalend is voor de gevoeligheid voor cisplatine (Hoofdstuk 3, 4 en 5), terwijl veranderingen in de niveaus van cytoplasmatisch p21 (Hoofdstuk 2) en een stringente regulering van p53 door MDM2 (Hoofdstuk 4) de resistentie tegen cisplatine kunnen verklaren. Daarnaast kan het expressie niveau van BLMH bepalend zijn voor de response op bleomycine (Hoofdstuk 6), een belangrijk en essentieel onderdeel van de op cisplatine gebaseerde chemotherapie welke wordt gebruikt in de behandeling van testiskanker. In preklinische studies werd na modulatie van p53/MDM2 of p21 een verbeterde effectiviteit van cisplatine aangetoond. Het vertalen van deze resultaten naar de kliniek kan leiden tot nieuwe behandelingen voor (refractaire) zaadbalkanker, door de combinatie van conventionele chemotherapie en toepassing van MDM2 antagonisten of PI3K/Akt remmers, om zo pro-apoptotische eiwitten te induceren of anti-apoptotische eiwitten te realloceren waardoor apoptose niet meer geremd wordt.

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Roelof